Biological control of Californian thistle (*Cirsium arvense*) with the rust fungus (*Puccinia punctiformis*)

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Cirsium arvense, commonly known as Californian thistle, is a problematic weed in New Zealand and around the world. In agricultural systems, *C. arvense* affects animal health and severely decreases plant yield. *C. arvense* is a highly invasive weed due to its creeping root system. This root system can be difficult for herbicides to control because it does not completely kill the roots, which overwinter in the soil, producing shoots in the spring. *Puccinia punctiformis* is a rust fungus that only infects *C. arvense. P. punctiformis* has the potential to be a successful biological control agent as it is already present in New Zealand and can systemically and locally infect *C. arvense.* The systemic infection by the rust fungus damages the roots and reduces shoot abundance in the weed. The aim of this research was to determine if the observed differences in the levels of infection in C. *arvense* populations within New Zealand was due to genetic differences between *P*. *punctiformis* isolates.

A survey of 22 sites in the North and South Islands of New Zealand was undertaken to determine the percentage of *P. punctiformis* infected *C. arvense* shoots in a designated area. *Puccinia punctiformis* was found in all 22 populations surveyed and the amount of *P. punctiformis* infected shoots varied between all sites. The Ruakura site had the highest amount of *P. punctiformis* infected shoots (11.1%), which was significantly higher than 15 other sites. Genetic analysis of the *P. punctiformis* isolates from some of the survey sites was undertaken by PCR. Sequencing of the ITS region indicated only one isolate (Hurst) out of 11 sequenced differed genetically (6 bp substitution). Comparatively, polymorphisms were observed with RAPD amplification which indicated genetic diversity across the whole genome for samples within both the population and between populations. Quantification of *P. punctiformis in planta* by qPCR determined that rust levels were at higher concentrations at the top of the plant and decreased to the base.

A glasshouse study was carried out to facilitate infection in order to increase the amount of inoculum for further work. No discernible infection methods were obtained. Only three out of the 32 treated plants had any infection, thus, considerable future work needs to be undertaken for a better infection method to be obtained.

The study has highlighted the difficulties in dealing with *P. punctiformis* which infects *C. arvense.* Infection in the field and the glasshouse is limited and variable. The study has indicated there is genetic diversity in *P. punctiformis* in New Zealand which could explain the observed differences in infection of *C. arvense* in the field.

Keywords: *Puccinia punctiformis*, *Cirsium arvense*, infection, genetic, diversity

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Introduction

1.1 Biological Control

Biological control of weeds uses natural enemies of an unwanted organism to damage and kill or significantly reduce the population of the unwanted plant. There are different types of biological control agents including insects, fungi and viruses or a combination of these to damage and/or kill the pest. The best use for biological control is implementing the method into an integrated pest management strategy which includes all control strategies, chemical, cultural, physical and biological to reduce a pest population (Froude, 2002; Hayes et al., 2013; Paynter, 2008). It can be a cost-effective way of controlling weeds, especially if classical biological control is applied. Classical biological control is a permanent solution and therefore agents should only be released once, unlike chemicals where it is an annual application to control weeds. Biological control is less damaging to the surrounding environment and communities compared to chemicals (Froude, 2002; Hayes et al., 2013; Morin et al., 2009).

Before agents are considered for biological control, the selection of the pest candidate is important. The weed needs to be either economically or ecologically important to be considered for a biological control programme. The weeds impact, distribution, biology and natural enemies are important factors when choosing a possible biological control candidate. The pests' natural enemies in the invaded area should be surveyed to know what ones are present in the area. Consideration into the natural enemies used to target the pest and what type of biological control the agent will be (augmentative, classical or inundative) will influence the biological agent candidate (Morin et al., 2009; Paynter, 2008). The agents' host range is extremely important as release into areas where it may result in non-target impacts, especially plants that are wanted, can be detrimental. Whether the crop is of economic or environmental importance will influence the type of biological control used (van Driesche et al., 2008).

The type of biological control programme used is dependent on the population of the weed and the agents available. The different types of biological control include classical, augmentative and inundative. Classical biological control is using the pests' natural enemies from its native range to control the pest population in its introduced range. The agent establishes and self-perpetuates resulting in a permanent solution to the weed population (Briese & Sindel, 2000; Culliney, 2005; van Driesche et al., 2008). Comparatively, inundative control method uses 'mass application', by applying large amounts of inoculum to the weed population creating a fast and high level of epidemic,

therefore needs the ability to be mass produced, survive in storage and transport. Application of inundative biological control agents is done when conditions are favourable for disease development and to achieve subsequent weed control. This method can include bioherbicides and mycoherbicides (Bellgard, 2008; Charudattan, 1988; Hajek, 2004). Augmentative biological control is when a biocontrol agent is present but only in a small number and causing minimal damage to the weed population and therefore spot application of the agent is applied. Augmentative biological control are supplemental additions to either classical or inundative biological control (Hajek, 2004; Van Lenteren & Bueno, 2003).

The classical control method is perhaps more cost-effective in the long run compared to augmentative and inundative because it is more of a permanent solution and only needs to be applied once if the agent establishes itself (van Driesche et al., 2008). The other two control methods are more immediate solutions compared to classical. Biological control can be an effective alternative to chemical use such as herbicides because it delays the effects of herbicide resistance which is a growing concern and is more environmentally friendly compared to chemicals (Hayes et al., 2013; Morin et al., 2009).

1.1.1 History and success of biological control

Research in the biological control area of weeds in New Zealand began in 1925 at the Cawthron Institute in Nelson. For a successful classical biocontrol, the agent needs to establish, however with augmentative and inundative biological control, this is not the case (Hajek, 2004; van Driesche et al., 2008).The release of biocontrol agents in New Zealand occurred as early as 1929, with the agent *Tyria jacobeae* targeting *Jacobaea vulgaris,* which successfully established as a self-perpetuating population (Froude, 2002; Hayes et al., 2013). Between 1931 and 1965 only nine biocontrol agents were released and two of those failed to establish. During this time biological control research faded and not many agents were released or studied because of the increase of new herbicides which were both effective and popular (Fowler et al., 2010; Froude, 2002; Hayes et al., 2013). In the early 1970s the use of herbicides began to slowly decline due to the growing disillusionment, which led to the increase in interest in biocontrol again, which has continued to date. The main weeds that were targeted throughout the 1970s to the late 1990s included ragwort and gorse for a second time, hawkweed (*Pilosella* spp.), broom (*Cytisus scoparius*) and some thistles such as Californian (*Cirsium arvense*) and nodding (*Carduus nutans*). At this time 28 agents were released which also included the first fungal pathogens. Biocontrol agents continued to be released throughout the following years. With nearly 90 years of biocontrol in New Zealand, 38 agents have been released and established against 17 targets. The number of biological control agents that have been released has grown in the

last few decades with the highest numbers released in the 1990s (Figure 1.1) (Fowler et al., 2010; Froude, 2002; Hayes et al., 2013).

Figure 1.1: Weed biocontrol released in New Zealand and species first released elsewhere since the 1920s (only the first release per species is counted) (Fowler et al., 2010; Hayes et al., 2013).

Biological control has increased in importance over the years for several reasons. Perhaps the most common and important reason is because of the growing concern of the development of herbicide resistance in weeds. Synthetic herbicides have been extensively used globally as it is one of the most common, effective and easy methods for controlling weeds. The development of herbicide resistance in weeds needs to be minimised and delayed because weeds impact the fitness of crops (Demers et al., 2006; Hayes et al., 2013; Morin et al., 2009). Herbicide resistance in weeds is growing due to the overuse and misuse of herbicides. Some plants have developed and evolved to be able to survive against certain herbicides and to be able to reproduce passing on the resistant gene to the offspring, making it more difficult to control weed populations. Herbicides are separated into different groups depending on the active ingredient and the different mode of action in which the herbicide kills the weeds. The weeds then develop resistance against that mode of action and the herbicides which use the same pathway. Weeds were mainly resistant to herbicides' photosynthesis inhibitors (Photosystem ll (PSll)), until around 2000 where there was a rapid increase in weeds resistant to herbicides that inhibit acetolactate synthase (ALS). Weed resistance to ALS inhibitors first appeared in 1985, and by 2014, 155 weed species were resistant to this group. While resistance to the other modes of action are not as severe as these two groups, they are still increasing in the number of species resistant to the other groups of herbicides (Heap, 2015). Resistance to herbicides has been

seen in some weeds in New Zealand. In the 1970s fathen (*Chenopodium album*) and willow weed (*Persicaria persicaria)* in maize crops developed a resistance to herbicides in the triazines group (Popay, 2016). More recently there has been an increase in the development of herbicide resistance to glyphosphate especially in ryegrass (*Lolium* spp.) in vineyards. Three populations of perennial ryegrass (*Lolium perenne*) and two populations of Italian ryegrass (*L. multiflorum*) have developed resistance to glyphosphate (Ghanizadeh et al., 2016). Another reason for the use of an alternative method for chemicals is because of the damage to the environment and the surrounding communities (van Driesche et al., 2008).

1.2 Classical Biological Control

Classical biological control is the introduction of natural enemies to the weed population to reduce the abundance of the weed. Classical biological control is generally used to control weeds over a large area and for long-term effectiveness, giving a permanent solution. When a plant is introduced outside its native range it often becomes a problem as their natural enemies are not present. The weed's natural enemy is then introduced to control the weed (Briese & Sindel, 2000; van Driesche et al., 2008). There are many key features of classical biological control that makes it an effective and sustainable method. These agents are selected for their ability to permanently establish and spread, therefore once achieved no more action should be required. This method may not be an immediate solution as it generally requires government support for an extended application period which can take 5-20 years for weed targets and 5-10 years for insect targets (Briese & Sindel, 2000; Culliney, 2005; Hayes et al., 2013; van Driesche et al., 2008). There are several examples of classical biological control in New Zealand which have successfully established themselves throughout the country or in parts of the country. The blackberry rust (*Phragmidium violaceum*) attacks blackberry (*Rubus fruticosis* agg.); the agent is widely established throughout the North and South Island of New Zealand (Hayes, 2005). Another highly successful classical biocontrol programme is the control of mist flower (*Ageratina riparia*) with a white smut fungus (*Entyloma ageratinae*) and a gall fly (*Procecidochares alani*) (Fowler, 2007).

There are many factors to consider when choosing a classical biological control agent including the spread to ecological limits of the agent as well as it geographical spread. The agent's population is able to spread naturally to new areas until they either reach a geographical barrier or their ecological limit. Agents reaching their ecological limit is normal, therefore understanding and predicting the range the agents will reach geographically is important because it will give a better understanding as to the areas the agent will invade and if any potential non-target species may be present in these areas (Hayes et al., 2013; Louda et al., 2003; Morin et al., 2009; Suckling & Sforza, 2014). Classical biological control has the potential to have high levels of control dependent on the success of the

agent and its ability to establish and spread. The speed as to which the agent has an impact on the weed plays a role in how successful an agent will be. The agent is generally released in small numbers compared to that of the weed population; the ability of the agent to reproduce and continue to reproduce for many generations is necessary for the agent to have an impact on the weed population. The time required for visible effects on a weed population can take a long period of time, from years to decades for the agent to reproduce and increase its population to be large enough to start having a significant effect on the weed population (Briese & Sindel, 2000; Morin et al., 2009; van Driesche et al., 2008).

There are several advantages and disadvantages of using classical biological control. Advantages include the permanent control of a pest with a natural enemy which is both environmentally friendly and acceptable to the surrounding communities. It can be more cost-effective in the long run because once the agent has been released and has successfully established no more applications should be required. The biological control agent can distribute itself throughout the weeds' populations across a wide area including area where human access is difficult (Culliney, 2005). Disadvantages of classical biological control include that it is not an immediate solution for controlling weeds; classical agents can take years to decades to be released and establish (Briese & Sindel, 2000; Suckling & Sforza, 2014; van Driesche et al., 2008). There are different types of non-target effects and risks that can be caused by the release of classical biological agents, such as important plants and crops being attacked. Non-target effects can be indirect and the impact level may vary thus testing the agents' host specificity is important as it determines what non-target plants may be at risk (Cullen, 1997; Louda et al., 2003; Suckling & Sforza, 2014). There are several examples of non-target impacts, where agents have caused damage to plants. However in New Zealand there has not been deleterious damage. Examples include broom seed beetles (*Bruchidius villosus*) which attack tree lucerne (*Cytisus proliferus*) seed. This non-target effect was not predicted (Paynter, 2008). Some other non-target effects have been predicted, but only to cause minor damage, these included Cinnabar moth (*Tyria jacobaeae*), introduced to control ragwort (*S. jacobaea*), where larvae sometimes attack native fireweeds *Senecio minimus* and *S. biserratus*. This generally occurred when the ragwort had been defoliated by the moth. Another example is Blackberry rust (*Phragmidium violaceum*) which was self-introduced to New Zealand and some minor damage has occurred on bush lawyer (*Rubus cissoides*) (Paynter, 2008).

1.3 Inundative biological control

Inundative biological control is applying large amounts of inoculum to a weed population to have a quick and effective control. This method is for the rapid control of pests, especially large populations, over a short period of time. This method can be used in areas for short-term control

where the natural enemies are unable to reproduce and survive in the habitat, generally, because the agent cannot survive in the conditions present or in the off season such as harsh winters (Bellgard, 2008; Hajek, 2004). Inundative control is similar to that of chemical control but more environmentally friendly. The agents released in this method are not expected to reproduce and establish and as such, application of the agent is required yearly which is similar to herbicides (Charudattan, 1988; Hajek, 2004). The agents are released and are expected to make contact and kill a high proportion of the targeted weed or at least cause major damage which results in the control of the weed population. For a sufficient amount of control, large amounts of inoculum needs to be applied which have similar effects to that of a disease epidemic (Hajek, 2004; van Lenteren et al., 2003). Damage is generally caused by the initial agent population and not the secondary infection. However it is often expected that some secondary disease does occur due to the secondary spread of the agents; the secondary spread does not cause the initial damage and control of the weed population (Charudattan, 1988; Hajek, 2004). Many factors need to be considered for the use of inundative control because unlike chemical storage, transport and mass production, bioherbicide production can be more difficult. Living organisms are used and therefore care needs to be considered when storing and transporting so they remain viable and able to infect the weeds. They also need to be released appropriately for the most effective infection and damage (Charudattan, 1988; Hajek, 2004).

Bioherbicides are generally used in this biological control method, it is similar to herbicides but with the natural enemies. Mycoherbicides are a type of bioherbicide with fungi as the active ingredient (Bellgard, 2008). One of the most common methods for the application of inundative biological control is with the use of bioherbicides and mycoherbicides. There are several known bioherbicides and mycoherbicides that have been registered throughout the world including, in Canada in 2007, *Sclerotinia minor* (mycoherbicide) targeting Dandelion (*Taraxacum officinale*) in lawns and turf, it is currently commercially available known as Sarritor. In New Zealand there is current research into the possibility and feasibility of several fungal pathogens for mycoherbicides including white soft rot (*Sclerotinia sclerotiorum*) for controlling Californian thistle (*Cirsium arvense*) and *Chondrostereum purpureum* for control against woody weeds such as black cherry (*Prunus serotina*) (Bellgard, 2008; Bourdôt et al., 2004). The advantages of this control method is the rapid control of large weed populations, similar to that of chemicals, but it is more environmentally friendly. The disadvantages of this method are the need for reapplication, often annually, of the agent to the target weed. The agent chosen for this method needs to be considered as not all natural enemies can easily be used for inudative control as mass production of the agent needs to occur which can be difficult. This method is not amenable to use with biotrophic fungi as they require a living host to survive and therefore are difficult to culture (Charudattan, 1988; Hajek, 2004). It can also be expensive with

yearly applications and mass production but it is a quick and effective method to rapidly decline a weed population. This method is more suited towards economically important crops and high value agriculture systems because it is more of an immediate solution compared to classical biological control (Charudattan, 1988; Hajek, 2004).

1.4 Augmentative biological control (Inoculative)

Augmentation or inoculative biological control is used when the pest population does not show an immediate threat but control is still required. Supplemental addition of natural enemies where the agent is already present but are scarce and need a small boost to increase the agent population (Hajek, 2004; Hoy, 2008). A 'spot application' of the biological control agent is applied, to a weed population where the agent is already present and needs to be replenished. This generally occurs when the conditions are favourable for the rapid increase in the weed and agent population. This method involves both the released agent and the secondary spread providing a long-term and self-sustainable control compared to inundative control. It is used in systems which require pest population control where a small amount of natural enemy is already present. Application of natural enemies can occur yearly or every few years to maintain an appropriate level of agent population to maintain a sufficient amount of control for the pest population (Hajek, 2004; Hoy, 2008). When the persistence of the agents' population is shortened due to the seasonal changes in an area the agent is released inoculatively each season, this is known as a seasonal inoculative release (Charudattan, 1988; Hajek, 2004; Hoy, 2008).

A common example of augmentative biological control agent is the inoculative release of the egg parasitoid *Trichgramma* wasps for the management of a wide range of Lepidoptera in various crops in many countries including Latin America and the USA (Mills et al., 2000; Van Lenteren & Bueno, 2003). *Trichoderma* spp. are common biological control agents against many different pests. It is a soil borne fungus that colonises roots of plants generally aiding its defence against pathogens. This agent sometimes needs to be applied seasonally due to plants being harvested which may result in the reduction of the agent. *Trichoderma* spp. have been reported to also aid in weed control of grass weeds and broadleaf weeds (Verma et al., 2007). There are several weeds and fungal agents that make good candidates for the augmentative control, these include *Puccinia canaliculata* of *Cyperus esculentus* in North American crops and in Chile *Phragmidium violaceum* for blackberry*, Rubus contricus* and *R. ulmifolius* (Charudattan, 1988, 2005; Sreerama Kumar et al., 2008). The advantages of this method is that it is a quick application method and can control the weed population relatively quickly unlike classical control. Similar to classical control it is environmentally friendly and safe to the surrounding communities. Because of its relatively low costs, this control strategy is preferred for weeds that are distributed over large areas that yield low or marginal economic returns

(Charudattan, 1988). Large amounts of the agent is not needed as it is only boosting the population which is already present. One disadvantages of this control include is that it can be difficult to apply to areas where it is difficult to access. The Augmentative method may require annual or seasonal application which may increase the cost of control (Hajek, 2004; Hoy, 2008).

1.5 *Cirsium arvense* (Californian thistle)

Cirsium arvense is commonly known as Californian thistle, creeping thistle and Canada thistle. *C. arvense* is a noxious, invasive weed of pastures and crops, commonly found in temperate regions throughout the world (Cripps et al., 2009). *C. arvense* is an extremely problematic weed in pasture systems, such as dairy systems and sheep and beef systems, reducing pasture yield and, therefore, reduce live weight gain of livestock reducing livestock production (Cripps et al., 2011; Eerens et al., 2002). In 2015 there were 6.5 million dairy cattle in New Zealand with exports earning in excess of $13.7 billion. The New Zealand dairy industry is mainly pasture based and dairy farming occupies 1.6 million hectares out of the total 12 million hectares of pastoral land. In all pasture systems, it is important to have effective weed control to maintain good pasture yield (DCANZ, 2016). Other than pasture, *C. arvense* is problematic in many crops including peas, wheat, beans and potato (Moore, 1975). The thistle competes with other plant species for light, nutrients and water, and this reduces crop yield (Moore, 1975).

1.5.1 *Cirsium arvense* Life Cycle

C. arvense is a perennial plant with an extensive, complex and strong creeping vegetative root system. It has two different cycles to survive and spread, the seed cycle and the vegetative cycle, with the vegetative cycle more problematic and difficult to control. *C. arvense* is a dioecious plant, it has separate male and female plants, which aids in genetic diversity allowing the thistle to better adapt to its different habitats (Moore, 1975). The male and female plants are in close proximity enabling pollination to occur via insects. However, this is not always the case as sometimes populations exist with clones of only one sex spreading via roots and no seed production. The two sexes can be easily distinguished from one another by the different flower heads in the late summer. Male flower heads are more globular and slightly smaller than the female which are more flask-shaped (Moore, 1975). The two sexes can also be distinguished by the presence of a well-developed stamen for male plants and the pistil in females (Moore, 1975). The seeds are small (Figure 1.2) and detach from the parent and blow to the ground where the majority of the seeds are then eaten by birds and other seed predators. The remaining seeds then germinate in spring in cultivated soil but not dense pastures or the seeds may germinate in dense pastures but may die due to competition by other plant species (Bourdôt, 2005; Lalonde & Roitberg, 1994). The seed cycle does not play an important role in the impact that *C. arvense* has in pastures because seedlings rarely increase the

weed population in the established pasture but they may contribute to the initial invasion and establishment into an area (Berner et al., 2013; Bourdôt, 2005). The vegetative cycle is the more problematic stage of *C. arvense* and causes the greatest impact (Berner et al., 2013). Once a seedling has established itself in an area it starts to develop a creeping root system and shoot buds. The roots continue to develop producing shoots throughout spring, summer and into autumn and can spread up to 2 m (Bourdôt, 2005; Lalonde & Roitberg, 1994). When winter comes, the above ground vegetation dies off, but one in five of the buds form subterranean shoots after dormancy. The shoots grow under the soil surface and emerge in spring forming a rosette, which then develops into aerial shoots (Berner et al., 2013; Lalonde & Roitberg, 1994). These new shoots continue to develop and the new roots creep underground while the previous seasons roots die off. The area where the thistle has established will continue to grow each season if no control measures are taken. If the roots are fragmented this promotes more spread as the root fragments release additional buds after dormancy (Bourdôt, 2005; Lalonde & Roitberg, 1994).

Figure 1.2: *Cirsium arvense* seed (Hurst, 2015).

1.6 *Cirsium arvense* Control Methods

1.6.1 Chemical control

Chemical control is a common and effective method for controlling weeds including *C. arvense*. Glyphosate herbicide is one of the more common herbicides used for the control of *C. arvense*; it can be applied by three different methods, including boom spray, handgun and knapsack, all with different rates. Glyphosphate should be applied to actively growing plants after the development of flower buds (AGPRO, 2013b). There are also several other herbicides that are effective at controlling the weed, including clopyralid and MCPA. Clopyralid herbicides can be applied with two different

methods, broadcast and spot spray; these should be applied from rosette to early flower and retreatment may be required (AGPRO, 2013a). MCPA herbicide is applied at early flower bud stage but when plants are at a height of 30-40 cm. Chemical rotations should be integrated into the management strategy to delay the effects of herbicide resistance which is a growing concern around the world and in many plants (AGPRO, 2013c). There are several herbicides that can be translocated down to the root system such as aminopyralid and clopyralid. It is important for the herbicide to be applied at a time when the herbicide will be translocated to the roots and not applied with fast acting herbicides or the plant will die before time is allowed for translocation of chemicals to the roots (Jacobs et al., 2006). Several varying levels of resistance have been observed in different *C. arvense* ecotypes to different herbicides including 2,4-D and Amitrole when applied and different life stages, bloom stage and bud stage (Hodgson, 1970).

1.6.2 Cultural and physical control

There are several different cultural and physical control methods that have been known to be effective and a successful way of controlling *C. arvense*. Mowing is known to be an effective control method, as it reduces the photosynthetic ability of the thistle which reduces the formation of the underground roots. However, mowing can be laborious and costly. Control at multiple or hard to access sites can be difficult. Mowing and sheep grazing can be integrated together as mature sheep will immediately graze on the thistles after the population has been mown, however when the thistles start to dry out they are no longer palatable (Bourdôt et al., 2016; Demers et al., 2006; Mitchell et al., 2002). Cutting thistles by any method such as mowing results in the root reserves being used for the regrowth of the weed and does not enable the plant to replenish them. When winter comes and the above ground vegetation dies off, there are not enough root reserves for the plant to successfully establish itself the following season (Hatcher & Melander, 2003). Mowing has also been seen to increase *Puccinia punctiformis* incidence in *C. arvense* sites compared to sites that thistles were not mown (Demers et al., 2006). There is also the suggestion that plant volatiles may be required for teliospore infection which is produced when the plant is damage such as mowing (French et al., 1994).

Cultivation is another method used to control *C. arvense* however this alone will not control the weed. Cultivation is part of an integrated control programme aiding in controlling large, well established thistle populations. Cultivation fragments the root system which then releases buds from dormancy and forces them to shoot. These shoots can then be controlled with other methods, such as chemicals, pulling and grazing early in the season. The weed needs to be under control and if possible eliminated before sowing of crops (Graglia et al., 2006). In pastures the types of species that are sown can have an influence on the growth and establishment of *C. arvense* (Graglia et al., 2006).

If plants with high competitiveness are sown then this will reduce the population of the thistle due to competing with nutrients (Graglia et al., 2006). In a study done by Graglia et al. (2006) it was found that presence of a grass and white clover mixture or red clover has an impact on the above ground biomass of *C. arvense* an thistle. The biomass decrease was thought to be caused by the suppression of the regrowth of the shoots after mowing or hoeing treatments in the late summer and autumn period. The increased interspecific plant competition has been found to reduce the abundance of *C. arvense* (Graglia et al., 2006).

1.6.3 Biological Control

In New Zealand there have been five agents released to target *C. arvense*. The Californian thistle flea beetle (*Altica carduorum*) that was released in 1979 and 1990 did not establish in New Zealand. It prefers to feed on the foliage and was found only to cause serious damage to *C. arvense* at high densities of the beetle (Cripps et al., 2011). *Hadroplontus litura* was also released against *C. arvense* but did not establish (Cripps et al., 2011). Californian thistle gall fly (*Uraphora cardui*) is limited by stock grazing. The gall fly females lay eggs in the terminal and lateral shoots of the *C. arvense*. When the eggs hatch about a week later, they burrow into the stem tissue feeding on the plant, the plant produces a gall which is swollen hard tissue. Swelling increases as the larvae grow and it harms the weed by diverting plant nutrients to the gall and decreasing nutrients for plant growth and development as well as root reserves. The galls in the terminal shoots are more damaging to the plant then the ones in the lateral shoots because they stop production of buds and significantly reduce stem height (Hayes, 2007a). Californian thistle leaf beetle (*Lema cyanella*) remains rare and is unlikely to cause a large impact to the thistle population (Hayes, 2007b). Both the adults and larvae feed on the leaves and stems of the weed which can cause a large amount of damage. During the summer the leaves have holes or areas where the membrane is very thin (Hayes, 2007b). More recently the green thistle beetle (*Cassida rubiginosa*) was released in 2007 (Cripps et al., 2011; Gourlay, 2010b) with the larvae and adults causing damage. The larvae feed and grow through five instars, the three youngest instars feed on the undersides of the leaves while the two oldest attack leaves from the top. The adults feed on the first thistles appearing in spring making holes in them (Gourlay, 2010b). The Californian thistle stem weevil (*Ceratapion onopordi*) was released in 2009 but has not established well (Cripps et al., 2011). The larvae, rather than the adult, cause the majority of the damage to the thistle. The larvae mine down into the *C. arvense* stems and roots killing the plant by reducing the weeds ability to compete with other plants (Cripps et al., 2009; Gourlay, 2010a; Wandeler & Bacher, 2006). This weevil generally attacks *C. arvense* when the rust, *P. punctiformis,* is present. It is possible the weevil may transmit the pathogen to other sites and promote systemic infection by *P. punctiformis,* which leads to the death of the stems (Gourlay, 2010a), however this is refuted (Cripps et al., 2009).

There are also several pathogens of *C. arvense* which could lead to potential biological controls. White soft rot (*Sclerotinia sclerotiorum*) causes water-soaked brown to black lesions generally near the base of the weed, which leads to the death of the weed. This pathogen has been found to cause more damage to the thistle if the plant has been wounded prior to infection, applied with a mycoherbicide (Bourdôt, Hurrell, & Saville, 2004). Phoma leaf blight (*Phoma exigua* var. *exigua*) produce chlorotic yellowing area turning to brown as the thistle starts to die. The Californian thistle rust (*P. punctiformis*) has been observed to damage the plants and be a potential biological control agent. Weeds that are infected with *P. punctiformis* are covered in yellow, orange or brown spores which leads to the weed becoming chlorotic and stunted, which results in the plant not being able to flower and the root buds being affected (Bourdôt, 2008; Landcare-Research, 2007).

1.7 *Puccinia punctiformis*

Puccinia punctiformis, commonly known as Californian thistle rust fungus, is native to Europe (Cripps et al., 2009; Waipara et al., 2009). *P. punctiformis* is a potential biological control agent for *C. arvense* and has several aspects which could make it a beneficial option. *P. punctiformis* is an autoecious fungus whereby it completes its lifecycle on the one host. It is host specific and only infects *C. arvense*. The rust fungus, although it self-perpetuates, has not caused epidemics in *C. arvense* populations in order to reduce the competitive ability of the weed to levels below the economic threshold (Johnston, 1990). *P. punctiformis* can infect a host localised or systemically. It is this systemic infection that has the potential to cause severe damage to the thistle. Systemically infected shoots which emerge are relatively tall, frail and deformed (Berner et al., 2013). If the fungus is able to grow systemically down into the roots and kill the roots, this would indicate potential as a biological control agent. It is not completely understood whether the fungus directly kills the roots, however, it is known to overwinter in the roots. In spring some shoots emerge from adventitious shoot buds that are systemically infected, resulting in *P. punctiformis* killing the shoots and thereby reduces subsequent root biomass, and potentially reduces thistle shoot abundance (Berner et al., 2013; Cripps, 2016; French & Lightfield, 1990). There are still gaps in the understanding of this fungus, in particular, whether or not infection is influenced by an interaction between isolates of *P. punctiformis* and genotypes of the host plant. Recent studies have increased our understanding of the *P. punctiformis* life cycle, however, the lack of epidemics caused by the fungus and subsequent control of *C. arvense,* has not been elucidated. There is still little known about the genetic diversity of *P. punctiformis*, comparatively, the plant is considered to be highly genetically diverse; whether or not this contributes to the virulence of the pathogen is unknown (Bommarco et al., 2010; Hettwer & Gerowitt, 2004). There is little understanding of the teliospores stage. It is thought to be the likely candidate in causing the systemic infection of *P. punctiformis* in the thistle (Berner et al., 2013; Cripps, 2016; Cripps et al., 2014; French & Lightfield, 1990).

1.7.1 *Puccinia punctiformis* Lifecycle

Puccinia punctiformis is a biotrophic fungus causing etiolation of systemically infected shoots which leads to the necrosis of the stem and leaves (Thomas et al., 1994). Stems that are infected may not flower and root buds may be affected (Bourdôt, 2008). *P. punctiformis* is a macrocyclic fungus where all five spore types are produced: spermatia, aeciospores, urediniospores, teliospores and basidiospores (Alexopoulos et al., 1996; Berner et al., 2015).

Plants that are infected with *P. punctiformis* are usually covered in bright orange coloured aeciospores. The aeciospores become urediniospores, that are an orange-brown in colour. Subsequently, the plants become chlorotic and appear stunted (Figure 1.3; (Berner et al., 2015)). This fungal pathogen overwinters as mycelium in the roots and when conditions become favourable at the start of spring, the shoots will emerge already systemically infected with the pathogen. Mycelium then develops, spreading and infecting throughout the plant producing spermagonia which eventually form the aeciospores with the most favourable temperature between 18°C-25°C. In late spring these spores spread and infect neighbouring plants producing local lesions, which then develop into uredinia bearing urediniospores causing further infection. In late summer the urediniospores transform into telia producing teliospores. By late summer and autumn the leaves of the thistle fall off, the teliospores then blow off onto newly emerged rosettes (Berner et al., 2013; Thomas et al., 1994). When conditions become favourable, temperatures are 5-15°C (Thomas et al., 1994) teliospores will germinate producing basidium bearing basidiospores. The fungus then grows hyphae and the mycelium will grow down into the roots (Anikster, 1986; Berner et al., 2013; Thomas et al., 1994). When the shoots emerge in spring they are infected with the overwintering fungus which turns into spermagonia which then transforms into aeciospores and subsequently urediniospores. The spring/summer cycle continues causing local infection. It can be difficult to distinguish between aeciospores and urediniospores, thus the spores type causing the most infection is unknown (Alexopoulos et al., 1996; Baka & Lösel, 1992b; Berner et al., 2013; Thomas et al., 1994). The disease life cycle (Figure 1.4) of this fungus is not yet completely understood to determine the cause of the systemic state of the disease (Berner et al., 2013).

Figure 1.3: *Puccinia punctiformis* symptoms on *Cirsium arvense* (Bourdôt, 2008; Nicholls, 2011).

Figure 1.4: Summary of a macrocyclic rust fungus life cycle, (Alexopoulos et al., 1996; Berner et al., 2015)

1.7.2 Advantages and disadvantages

There are many benefits of *P. punctiformis* as a biological control agent of *C. arvense*. *P. punctiformis* is highly host specific therefore it is safe as a biological control agent as it will not infect other non-target plants. *P. punctiformis* is present throughout the world in areas where *C. arvense* is present, and it can infect both locally and systemically. The systemic infection of the fungus has the potential to cause the most damage, and understanding the infection and the disease cycle associated with the systemic infection is important for manipulating greater damage (Berner et al., 2013). There is also potential to aid in the spread of this pathogen with other biocontrol agents such as the green thistle beetle which could enable it to spread to weed populations in other areas. This pathogen could be beneficial as it has the potential to work with other pests of *C. arvense* and work together to damage and kill the weed. Augmentation of *P. punctiformis* populations may be required to help facilitate its spread (Berner et al., 2015; Cripps et al., 2009).

There are also several disadvantages with of *P. punctiformis* as a biocontrol agent. Although the fungus is extremely host specific it may not infect all plant genotypes. This has been observed in many other biological control programmes including the Brazilian pepper tree, *Schinus terebinthifolius,* where insect biocontrol agents performed variably on different plant genotypes (Manrique et al., 2008). *P. punctiformis* is a biotrophic fungus requiring a living host to survive and reproduce, therefore it is unable to be cultured *in vitro*. This can make mass production difficult for the initial release more difficult (Thangavelu et al., 2004). The ability of this fungus to establish and spread into areas where *C. arvense* populations are located is important for the permanent classical biological control of the weed population. A biological control agents' ability to overcome landscape barriers (mountains, forests, rivers) is an important aspect. It may rely on other factors such as insects or human influence to help it overcome barriers. As *P. punctiformis* is present in *C. arvense* populations throughout New Zealand, this suggests the pathogen has not been hindered by such barriers which is not surprising as it is largely wind dispersed.

1.8 Molecular techniques

Molecular techniques are a common method for determining genetic differences between isolates and species. Molecular analysis can give an indication of possible genetic variability between isolates of *P. punctiformis* which may be attributable to differing levels of infection within populations of *C. arvense*. There are various molecular techniques that can be used to determine genetic differences. The various techniques include the Polymerase Chain Reaction (PCR), amplification of the Internal Transcribed Spacer (ITS) region, Restriction Fragment Length Polymorphism (RFLP), DNA sequencing, Random Amplified Polymorphic DNA (RAPD) and Quantitative PCR (qPCR) (Saikia & Kadoo, 2010).

PCR is a molecular technique that allows for the amplification of a single or few copies of the target DNA. Selection of specific primers that target the desired sequence is important as this allows the identification of specific genes or areas of the genome of the target (Gil-Lamaignere et al., 2003; Saikia & Kadoo, 2010). Once the products have been amplified, the products are then run through electrophoresis and exposed to UV light to visualise the banding pattern that is produced. Amplification of the Internal Transcribed Spacer (ITS) region enables the identification of genetic differences between species. The amplification of the ITS region uses specific primers that target this region (Gil-Lamaignere et al., 2003; Miranda et al., 2010). Products that are amplified during a PCR reaction can then be digested by enzymes this is known as Restriction Fragment Length Polymorphism (RFLP). This technique can sometimes be referred to as cleaved amplified polymorphic sequences (CAPS) (Rasmussen, 2012b). RFLP can be used to investigate intra- and inter-species variation by exploiting variations in homologous DNA sequences (Rasmussen, 2012b). Different banding patterns may be produced which may indicate genetic diversity. PCR products can also be sequenced which allows for a close analysis of the amplified sequence which gives close indication of genetic difference such as being able to identify nucleotide substitutions (Gil-Lamaignere et al., 2003; Rasmussen, 2012b).

Random amplified polymorphic DNA (RAPD) is another PCR-based tool for assessing the whole genome by amplifying the DNA at different points producing banding patterns that differ between genetically different individuals. RAPD PCR uses a single short primer for a random amplification under specific PCR conditions. RAPDs are multilocus markers and their mode of inheritance is dominant. The number of amplified fragments depends on the distribution and number of annealing sites throughout the genome (Feng et al., 2009; Noonan et al., 1996). Quantitative polymerase chain reaction (qPCR) or real-time PCR is a molecular technique based on the Polymerase Chain Reaction (PCR). Quantitative PCR can be used to quantify the amount of pathogen within a host using specific primers. When the DNA is amplified by the primers it is monitored during each cycle. The different levels of amplified DNA are detected by a fluorescent dye, most commonly SYBR Green (Arya et al., 2005). Therefore, the higher the concentration of DNA the more fluorescence and the earlier it crosses the threshold (Heid et al., 1996; Ramakers et al., 2003).

1.9 Aims and Objectives

The aim of this research is to determine if the observed differences in infection of *C. arvense* were attributable to genetic variation between *P. punctiformis* isolates in New Zealand.

The objectives of my research are as follows:

1. Survey and collection of *P. punctiformis* isolates on *C. arvense* populations across North and South Islands, New Zealand (Chapter 2).
2. Genetic characterisation of *P. punctiformis* isolates (Chapter 3).
3. Determine the concentration of *P. punctiformis in planta* (Chapter 4)
4. Determine the most effective method to infect *C. arvense* with *P. punctiformis* (Chapter 5).

Currently, there is a lack of knowledge on the genetic diversity of the biological control agent for *C. arvense,* the rust fungus, *P. punctiformis.* It was hoped the present study will help close the knowledge gap on the diversity of *P. punctiformis* in New Zealand and identify potential reasons for differing infection levels in the noxious weed, *C. arvense.*

Chapter 2

Puccinia punctiformis survey

2.1 Introduction

Cirsium arvense is an invasive weed to New Zealand and is highly problematic in agricultural systems. There are several control methods for this weed with herbicides most commonly used. However, herbicide control differs between locations and needs to be applied annually (Jacobs et al., 2006). A potential biological control agent is *Puccinia punctiformis,* commonly known as the Californian thistle rust fungus. It is present in New Zealand and only infects *C. arvense* but as with herbicide applications, control by the fungus differs between localities (Berner et al., 2015; Cripps et al., 2009).

Surveys are an important method in understanding the distribution and density of populations of species as it may give an insight into the climatic and habitat range of the plant, pathogen and their interaction. Surveying a wide range of areas is important as these can differ greatly in many aspects including weather conditions, topography and the type of grazing. Some of these site characteristics could influence species distribution and abundance. The spread of some fungal pathogens could be inhibited by the amount of wind activity, rainfall and the type of topography (Burdon et al., 1989; Nagarajan & Singh, 1990; Shaw & Osborne, 2011). *P. punctiformis* requires high relative humidity for germination and infection and therefore would be limited in dry areas. Wind could influence the spread and infection of *P. punctiformis* spreading to other populations and regions. *C. arvense* sites may be sheltered from various weather conditions influencing the amount of *P. punctiformis* infection (Berner et al., 2015; Bourdôt et al., 2016; Hardwick, 2002; Plantegenest et al., 2007). The topography could affect the percentage of infection and could be related to the weather conditions. The direction the site is facing may influence the amount of rust infection because it could be exposed to various weather conditions (Hardwick, 2002; Plantegenest et al., 2007). The density of *C. arvense* populations could influence the amount of rust infection occurring at a site. There have been studies that look at the density of plants and the interaction with pathogens and found it to be an important factor in plant disease. Dense populations could increase the amount of pathogen because there is an increased number of hosts for the pathogen to infect (Burdon & Chilvers, 1982; Burdon et al., 1989).

C. arvense has been studied for many years with surveys conducted assessing the distribution of the weed in countries including Australia, New Zealand, Canada and throughout Europe (Cripps et al., 2010; Moore, 1975; Weber & Gut, 2005). Several surveys have been conducted looking at the infection of *P. punctiformis* on *C. arvense* as well as some related species but the pathogen was only

found to infect *C. arvense* (Waipara et al., 2009). There have been some surveys looking at *P. punctiformis,* generally in conjunction with other pathogens. In a study, it was found that the levels of *P. punctiformis* were found to be similar in New Zealand to its native range in Europe (Cripps et al., 2009; Waipara et al., 2009).

The aim of this trial is to determine if there is any difference in *P. punctiformis* infection in a broad range of climatic regions and habitats of *C. arvense* populations around New Zealand and in addition to aiding in further experiments to determine if there are any genetic differences in *P. punctiformis* in New Zealand.

2.2 Materials and method

2.2.1 Surveying Method

Twenty-two sites around the North and South Island of New Zealand were surveyed (Figure 2.1). A population or patch size of 25 m × 25 m were surveyed to determine the percentage of rust infected shoots within a population. They were then compared with other sites throughout New Zealand. Each quadrat was 1 m × 1 m and located 10 m apart, a total of 9 quadrats (Figure 2.2). Within the quadrat, the number of total shoots were counted, noting the number of rust infected shoots. If there were no rust infected shoots within the quadrat then a 5 m radius from the quadrat was surveyed. The number of infected shoots were counted and the closest shoot was measured from the quadrat, and sampled to be taken to the laboratory for further processing. The population size and/or patch size was recorded along with the topography of the paddock, altitude, longitude, latitude, distribution and density of *C. arvense* and any other relevant characteristics of the site; these were not analysed but may aid in looking at the differences between sites.

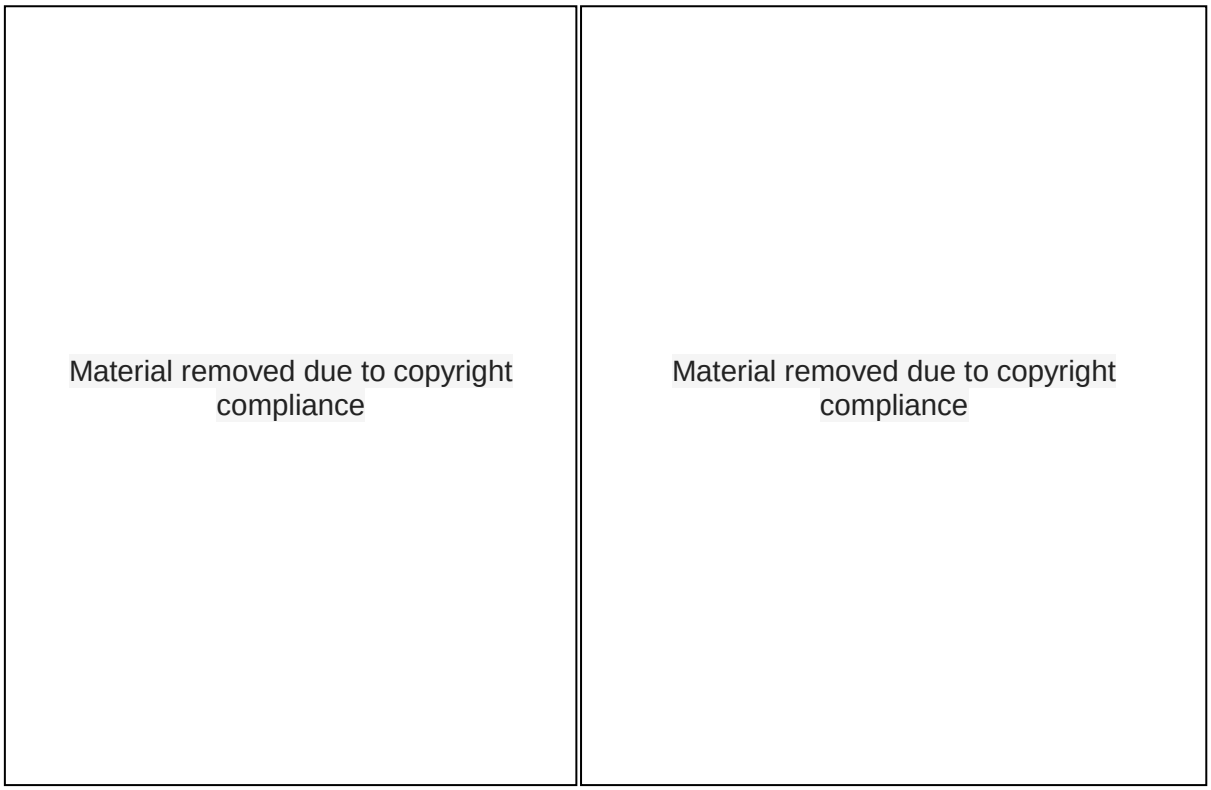

Figure 2.1: Survey of *Puccinia punctiformis* infecting *Cirsium arvense* at 22 sites in New Zealand in December 2016/January 2017. Fifteen sites in the North Island and seven in the South Island (Map data, 2018).

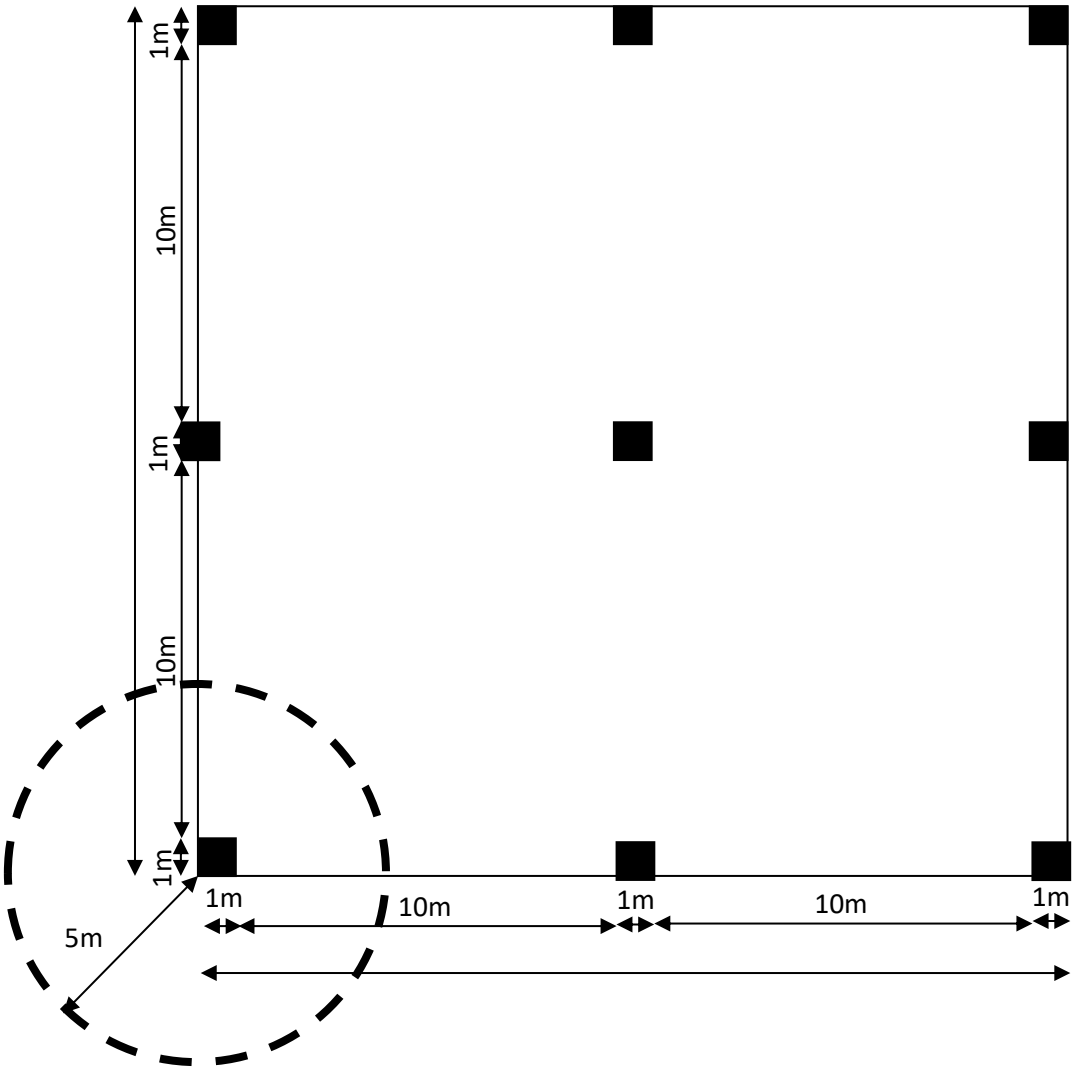

Figure 2.2: Survey method diagram at each site with *Puccinia punctiformis* infected *Cirsium arvense* plants. ■ is the sampling quadrats 1m x 1m. Dashed circle is the sampling area if no rust infected shoots are found within quadrat, 5m in diameter.

2.2.2 Sampling Method

Within the quadrat, all shoots infected with rust were collected, cut off at ground level and placed into a paper bag. If there were no rust infected shoots within the quadrat then the closest shoot within 5 m was cut at ground level and placed into a paper bag, recording the distance from the quadrat. Data was analysed using a generalised liner model (GLM) with binomial distribution through a logit function and then summarised using Duncan's lettering in Minitab version 16.

Table 2.1 Survey of *Puccinia punctiformis* infected *Cirsium arvense* sites throughout New Zealand. Indicated are the rust survey site number, name, region longitude, latitude and altitude.

#	Site	Region	Longitude	Latitude	Altitude (m)
1	Cussen Rd	Waikato	175.612393	-37.6455622	28.5
2	Ruakura	Waikato	175.317309	-37.7775708	42
3	Bridge 64	Waikato	175.834679	-37.9935509	109.5
4	Highlands 2	Bay of Plenty	176.384572	-38.2476292	547
5	Highlands 1	Bay of Plenty	176.367911	-38.2556964	585
6	Wi Pere	Gisborne	177.850087	-38.5646089	208.5
7	Manutuke	Gisborne	177.914308	-38.7133739	8
8	Wai-iti Rd	Hawkes Bay	177.774101	-39.012555	13
9	Poutu	Waikato	175.821409	-39.0567034	462
10	Duncans	Manawatu-Wanganui	175.920444	-39.6363731	491.5
11	Micklesons	Manawatu-Wanganui	175.812824	-39.6950433	454.5
12	Sheridon	Manawatu-Wanganui	175.355655	-40.0316794	185.5
13	Bushfield	Wellington	175.734902	-40.8022756	400
14	Bennet's Hill	Wellington	175.770794	-40.9657875	131.5
15	Gilbert's Rd	Wellington	175.193942	-41.0823342	228
16	Clyde	Canterbury	172.40076	-43.679973	10
17	Hurst	Canterbury	171.068616	-44.339138	196
18	McDonald	Canterbury	171.008665	-44.441442	139
19	Smith	Otago	170.631931	-44.962214	230
20	Dunrobin Valley	Southland	168.115902	-45.7359983	321.79
21	Milligans Rd	Southland	168.35812	-45.8982183	125.5
22	Happy Valley	Southland	167.7875	-46.123805	173.3

2.3 Results

All populations had rust present, although it was not necessarily detected within the quadrats of the sampling method. The Ruakura site had the highest percentage of rust infected shoots in the population at 11.1%, Manutuke was the second highest with 9.2% of rust infected shoots. Eight of the 22 sites surveyed had no rust infected shoots detected by the survey method (Figure 2.3). Populations with common letters do not significantly differ (P>0.05) (Figure 2.3). Analysing the percentage of rust infection from only the quadrats that contained *P. punctiformis* infected shoots still indicated that Ruakura still had the highest infection at 21.4%. The Clyde site also had a high

number of infected shoots with 20%, followed by Hurst at 14.3% (Figure 2.4). When only the quadrats with rust infected shoots were included in the analysis, Manutuke was 12.7% compared to 9.2% in all the population. The number of quadrats that had infected shoots differed between populations. Micklesons had seven quadrats with infected shoots while Ruakura had only three quadrats with infected shoots (Figure 2.5).

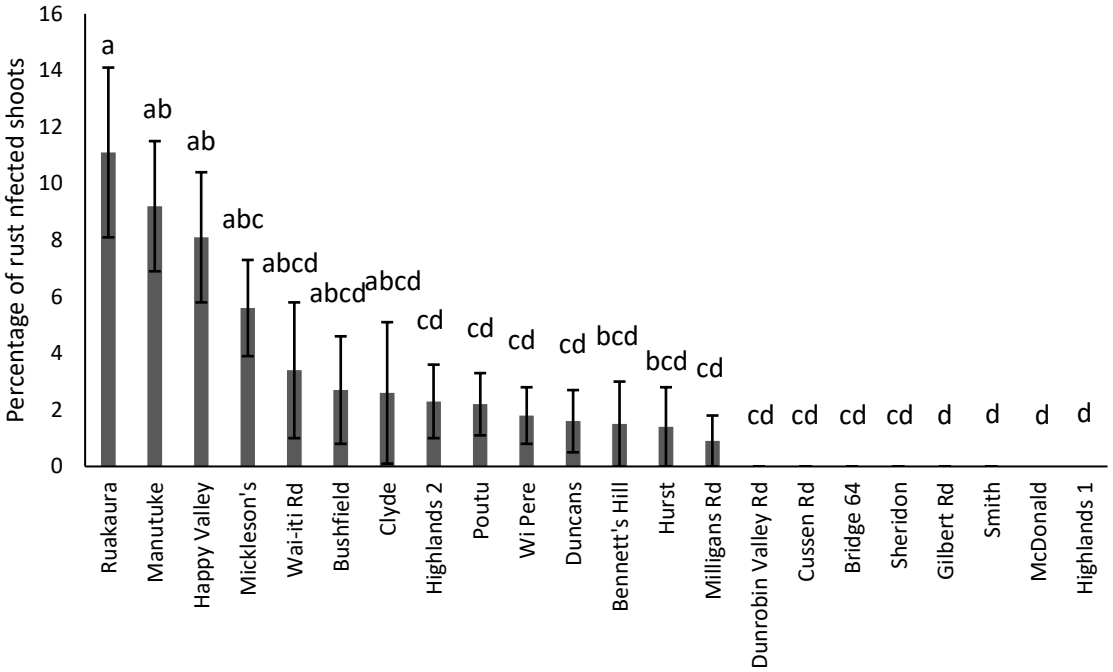

Figure 2.3: Mean percentage of *Puccinia punctiformis* infected shoots per *Cirsium arvense* population across New Zealand. Standard error bars of the mean are indicated (SEM). The Ruakura site had the highest percentage of rust infected shoots in the population at 11.1%.

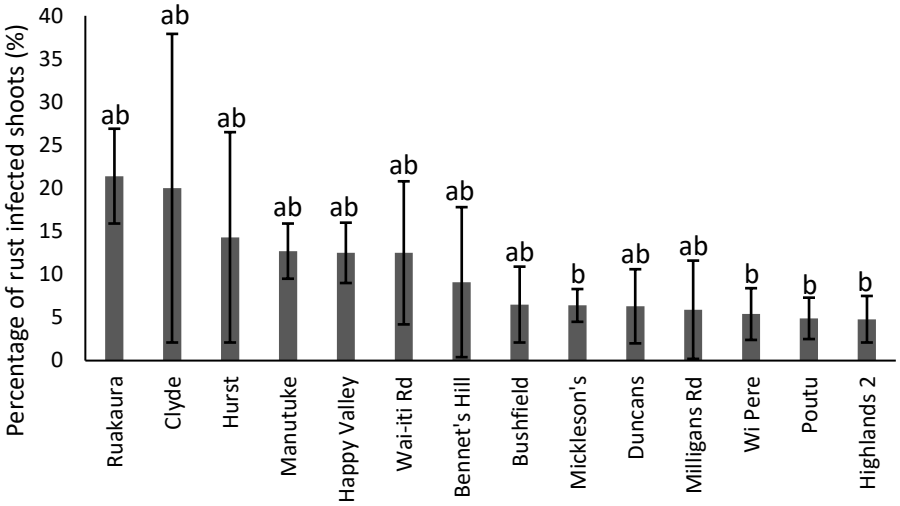

Figure 2.4: Percentage of *Puccinia punctiformis* infected *Cirsium arvense* shoots only in quadrats containing rust infected shoots per population. Standard error bars of the mean are indicated (SEM).

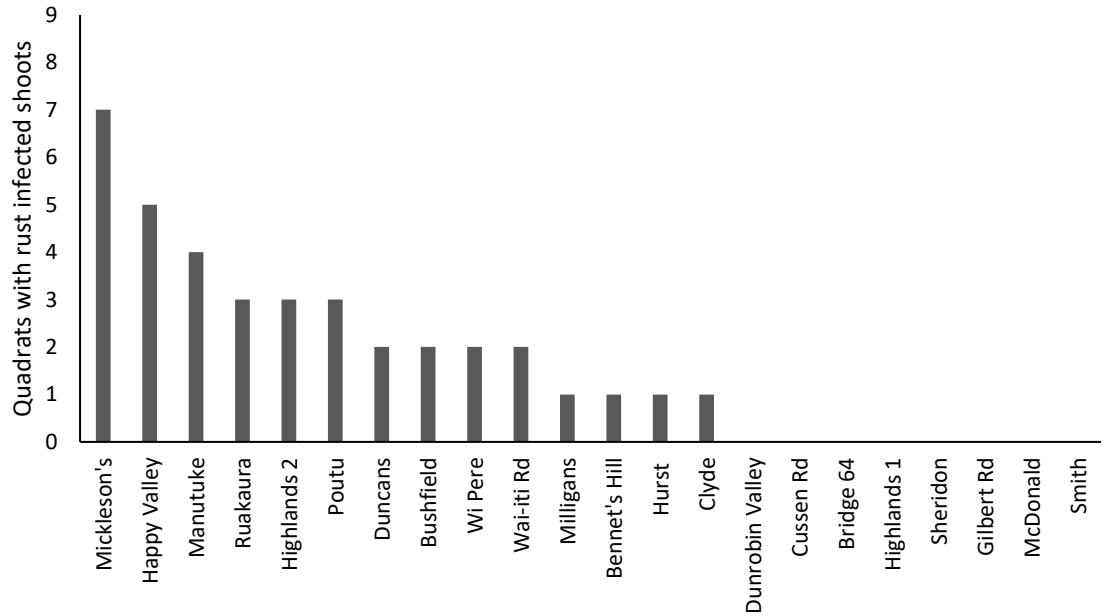

Figure 2.5: Number of quadrats (out of nine) with rust infected shoots in each population of *Cirsium arvense*.

2.4 Discussion

This survey gave an indication of the amount of *P. punctiformis* infected shoots in *C. arvense* populations across New Zealand. *P. punctiformis* was present in all *C. arvense* populations surveyed, however, the rust infected shoots were not necessarily in the quadrats. For all sites, the amount of infection varied and all had shoots that exhibited no disease symptoms. These results indicate the pathogen effectively reaches and subsequently infects *C. arvense* sites, as all populations surveyed had rust infected shoots. The visible amount of infection caused by *P. punctiformis* on *C. arvense* at each of the surveyed sites is not high and is variable within and between plants. The Ruakura site had the highest amount of rust infected shoots within the surveyed area (11.1%). The distribution of the rust infected shoots varied between the different sites, this can be seen with the number of quadrats with rust infected shoots. The Ruakura site had the highest amount of *P. punctiformis* infected shoots overall but had only three quadrats with rust infected shoots. The Micklesons site had the highest number of quadrats with rust infected shoots (7 quadrats) but only 5.6% of the surveyed population had *P. punctiformis* infected shoots. The method used in this study allowed for the identification of *P. punctiformis* within a given area but did not include all rust infected shoots within the populations, thus an underestimation of rust infection may have occurred. From the overview of the sites it appeared that the *P. punctiformis* infected shoots were clustered compared to being evenly spread throughout the population; some infected shoots were juxtaposed to non-symptomatic shoots. This survey has highlighted the sporadic nature of infection within a *C. arvense* population. This could be due to several reasons including the *C. arvense* activating a plant defence mechanism that prevents the pathogen from growing and spreading to the surrounding plants (Agrios, 2005). It has been found that *C. arvense* has various degrees of quantitative resistance amongst clones, this may be another factor affecting the number of shoots infected within a population (Bommarco et al., 2010; Hettwer & Gerowitt, 2004).

There were varying levels of rust infected shoots between all sites. The Ruakura site possessed plants that had no visible infection or ramets that were only partially infected; this was also observed at other sites. The characteristics of a site such as topography could influence the spread and infection of the pathogen within and between populations. The prevailing direction of the *C. arvense* populations could influence the spread of *P. punctiformis*, as well as the subsequent recruitment of *C. arvense*. The flat open areas could be expected to get higher amounts of rust infection because the pathogen could come from any direction and is more exposed to the weather conditions. This could be seen with the Ruakura site which was a flat paddock with houses nearby, and the *C. arvense* population was described as evenly distributed with a few patches; this site had the highest levels of rust infected shoots surveyed (11.1%). However, the Wi Pere site was in a flat paddock with trees and a stockyard nearby similar to the Ruakura but had lower amounts of rust infected shoots (1.8%).

The level *P. punctiformis* infection may differ on hills as the population may be protected from one side. The pathogen may only reach the *C. arvense* population from the direction the hill is facing and the site may be prone to certain weather conditions. North facing populations are more exposed to UV (ultra violet) light; it is known that all spore types of *P. punctiformis* are sensitive to light and therefore would be expected to have lower amounts of *P. punctiformis* infection (Subrahmanyam et al., 1988; Turner et al., 1986). This can be seen in sites Highlands 2 (2.3%) and Hurst (1.4%) unlike the North facing Happy Valley site where there are high levels of rust infected shoots (8.1%). The Happy Valley site may have higher levels of infection compared Highlands 2 and Hurst because Happy Valley is in Southland where conditions are generally cooler and wetter compared to Hurst which is located in Canterbury, where it is known to be dry and hotter than Southland (Caloiero, 2014). All these varying conditions, exposure to UV, dryness and heat could limit the amount of infection which is seen in the Hurst site (Anikster, 1986; Berner et al., 2013; French & Lightfield, 1990; Subrahmanyam et al., 1988). The Happy Valley site was in a slight gully which may shelter the *C. arvense* population from various weather conditions. Generally, in a gully, there will be longer dew periods in the morning and hence greater amount of time in higher relative humidity which may result in greater infection (Berner et al., 2015; Morin et al., 1992). The McDonald site was located in a gully and had very low amounts of rust infected shoots, which suggests that perhaps sites in sheltered areas may struggle to get new *P. punctiformis* infection from outside areas as well as spread within the population due to the lack of air movement that may occur. The Micklesons site is protected from one direction due a forestry block, this site still has high amounts of *P. punctiformis* infection. In this survey, the sites had varying climatic and habitat ranges but between the different sites there were similarities and differences, therefore it is difficult to obtain as to why some sites had higher amounts of rust infected shoots compared to other sites.

Other factors that may affect the amount of *P. punctiformis* infected shoots, could include sheep grazing within a population. Sheep have been seen to graze on *P. punctiformis* infected shoots because it is thought they are softer for the sheep's mouth and the rust has a floral smell which may attract the sheep (Bourdôt et al., 2016; Bourdôt et al., 2004). The grazing could simulate mowing, and mowing has been seen to increase the incidence of *P. punctiformis* infection in a population (Bourdôt et al., 2016; Bourdôt et al. (Bourdôt et al., 2016; Bourdôt et al., 2004; Tiley, 2010); whether this occurs with sheep or not is unknown. In this survey, the Ruakura site and the Highlands 2 site had no grazing in the population, these two sites had variable amounts of rust infected shoots within the population. The Manutuke site and the Wi Pere site were both grazed by sheep, the Manutuke (9.2%) site had higher amounts of *P. punctiformis* infected shoots compared the Wi Pere site (1.8%). The sheep density and grazing was not analysed in this study so it is unknown whether sheep grazing has an effect on *P. punctiformis* infection but it appears to have little effect as grazed and non-grazed

sites were spread through the data set. Further studies could look at different factors that may influence the spread and infection of rust within and between *C. arvense* populations.

Another potential, and highly probable reason there were differences in rust infection, is due to genetic differences in *C. arvense* between and within populations (Bommarco et al., 2010; Hettwer & Gerowitt, 2004). This genetic diversity could explain why there is such a variation in the level of *P. punctiformis* infected shoots in different populations. Genetic characterisation of *C. arvense* at the survey site was not undertaken in this study, so it is unknown whether the populations are clones or are genetically different. It is known that *C. arvense* has a wide range of clonal differentiation within populations (Bommarco et al., 2010; Hettwer & Gerowitt, 2004). Differences in the genotype would influence the virulence of *P. punctiformis* at the sites and be expressed in varying levels of rust infection; this was observed in this study. This has been noted previously with differences in susceptibility to the bioherbicide, *Sclerotinia sclerotiorum,* which was equated to genotypic differences in *C. arvense* populations (Smith et al., 2016).

The levels of *P. punctiformis* was found to be similar in New Zealand to its native range in Europe (Cripps et al., 2009; Waipara et al., 2009). Both the North Island and the South Island were surveyed which gives a variety of different climatic regions and habitats, 22 sites in total were surveyed. The West Coast and top of the South Island and the West and top of the North Island regions were not surveyed. The West Coast of the South Island has a higher rainfall than most of New Zealand (Caloiero, 2014) and therefore may increase the incidence of rust infection because rainfall is thought to increase rust infection especially after mowing (Bourdôt et al., 2016; Bourdôt et al., 2004). However, due to the higher rainfall, *C. arvense* is not as problematic in the West Coast compared to other weeds and areas (Moore, 1975). The top of the North Island may be a better climate for both the thistle and *P. punctiformis,* therefore may have an increase in rust infected shoots whether this is due to larger thistle populations or better conditions for infection (Anikster, 1986; Berner et al., 2013). The highest infected site was the second most northern site which may indicate that this is a possibility, however the most northern site Cussen Rd had lower amounts of *P. punctiformis* infected shoots compared to the Ruakura site.

The *C. arvense* rust survey was conducted over the 2016/2017 Summer months, from the end of November until the end of January. The timing of the year the populations were surveyed may change the amount of rust present at the different sites. There were large breaks between the first and last samples collected. The first samples were collected in Southland, at the end of Spring when plants generally start to show symptoms. Plants that were not collected, had no visible signs of infection, but may have been asymptomatic (Berner et al., 2015). The last samples were collected in Canterbury at the end of summer leading into Autumn. This time is predominantly where pustules

are present and visible on plants making it easier to identify *P. punctiformis* infection. There is always a possibility that plant were infected but were asymptomatic. More intensive surveys will allow for a more accurate percentage of rust infected shoots to be calculated while also collecting asymptomatic plants and testing them for the presence of *P. punctiformis* by molecular methods. It may also be beneficial to sample roots. Presence of *P. punctiformis* within the roots generally indicates that the pathogen has overwintered in the roots and will most likely spread throughout the plant (Berner et al., 2015; French & Lightfield, 1990). A more intensive survey will allow for the genetic diversity of *C. arvense* to be undertaken by molecular means which may give an indication as to why there are different levels of *P. punctiformis* infection in *C. arvense* populations across New Zealand.

2.5 Conclusion

This survey looked at the current percentage of *P. punctiformis* infected shoots in 22 *C. arvense* populations across New Zealand in the North and South Island. *P. punctiformis* was present in all sites surveyed. There were differences between the amount of rust infected shoots in *C. arvense* populations in New Zealand. This survey study had similar results to those found in other studies both in Europe and New Zealand (Cripps et al., 2009). There could be many factors that affect the amount of *P. punctiformis* infected shoots within *C. arvense* population. There does not appear to be any differences in levels of infection compared to the native range. This suggests that *P. punctiformis* does not have high levels of infection in *C. arvense* populations even in its native range. All the sites have some similarities and differences that gave no indication as to why some sites had higher levels of *P. punctiformis* infection compared to other sites. More intensive surveys could be conducted to obtain a more detailed survey on the percentage of rust infected shoots because some shoots may be asymptomatic. These studies could then lead onto looking at the genetic diversity of *P. punctiformis*, because it is still unknown whether *P. punctiformis* is highly genetically diverse and whether this contributes to the virulence of the pathogen.

Chapter 3

Puccinia punctiformis isolate identification

3.1 Introduction

Cirsium arvense is a highly problematic weed in New Zealand. Many studies have investigated *C. arvense* population distribution, lifecycle, pathogen-host interaction and genetic diversity (Bommarco et al., 2010; Hettwer & Gerowitt, 2004). *C. arvense* is genetically diverse and is known to have a wide range of clonal differentiation suggesting sexual reproduction occurs in populations (Bommarco et al., 2010; Hettwer & Gerowitt, 2004). *P. punctiformis* is a biotrophic fungus and is highly host specific completing all stages of its lifecycle on *C. arvense. P. punctiformis* is observed in many *C. arvense* populations, however its infection levels differ between populations; why this occurs is unclear (Demers et al., 2006; Frantzen, 1994). The genetic diversity of *P. punctiformis* is unknown and whether this contributes to the variability of the pathogen's infection of *C. arvense* has not been elucidated. It is likely there is genetic variation in *P. punctiformis* as this is observed in other *Puccinia* species. These species include, but are not limited to *P. striiformis* (heteroecious), stripe rust of wheat, *P. graminis*, stem rust (heteroecious) (Kolmer et al., 2012; Kolmer & Ordoñez, 2007) and other types of rust species including the blackberry rust, *Phragmidium violaceum* (autoecious) (Gomez et al., 2008; Kolmer et al., 2012; Kolmer & Ordoñez, 2007).

Molecular techniques are a common method to assess the genetic diversity between isolates. Amplification of the internal transcribed spacer (ITS) region is utilised to identify differences between fungal species (Gil-Lamaignere et al., 2003; Karp et al., 1996). Specific primers have been developed by Berner et al. (2015) to amplify a 460 base pair product of the ITS region in *Puccinia* species. This amplified ITS-PCR product can undergo restriction fragment length polymorphism (RFLP) (Rasmussen 2012). RFLP can be used to investigate intra- and inter-species differences by exploiting variations in homologous DNA sequences (Rasmussen 2012). PCR products can be sequenced which allows a close investigation into the base pairs and identification of nucleotide substitutions. Random amplified polymorphic DNA (RAPD) is another PCR-based tool for assessing the whole genome by amplifying various fragments producing banding patterns that identify genetically different individuals. RAPD PCR uses a small single primer for random amplification under specific PCR conditions. The amount of amplified fragments depends on the distribution and number of annealing sites throughout the genome (Gil-Lamaignere et al., 2003; Karp et al., 1996).

The aim of this study was to assess the genetic diversity of *P. punctiformis* throughout New Zealand using molecular techniques.

3.2 Material and methods

3.2.1 Spore removal

Spores were removed from 180 plants that were collected during the survey at 22 sites around New Zealand (see Chapter 2 for detailed methods). Spore removal from the thistle leaves was done by one of three methods, dependent on the age of the plant material. Leaves that were 0-2 weeks after harvest had spores dusted off with a paint brush into a petri dish. The spores were then placed in 1.7ml tubes ready for extraction. Leaves older than two weeks and those that were more desiccated, were soaked in 3ml of water and two drops of Tween 20 (Labchem) solution. The spores dislodged from the plant surface into the aqueous solution were placed in 1.7ml tubes and centrifuged (for 10 s at 13,000 x g) for easy removal of spores for DNA extraction. For plants where spore removal was difficult, small sections (1cm^2) containing pustules were cut and the material underwent direct DNA extraction.

3.2.2 DNA extraction

Chelex 100 (BioRad) was used to extract DNA from the spore samples from 3.2.1. Spores were added to a 300 µl aliquot of 10% (w/v) Chelex dependent on the spore removal method (Section 3.2.1) either: 15-50 mg of spores, 50µl of spore solution or 1 leaf section. Tubes were vortexed for 10 s three times and placed into a heating block at 100°C for 10 min. Tubes were removed and vortexed for a further 10 s three times, then returned to the heating block for 10 min at 100°C. In total tubes were boiled for 20 minutes. After this time, tubes were centrifuged for 10 min at 13,000 × g. The supernatant (150µl) was removed and placed into 0.6 ml tubes. DNA concentration was measured using a Nanodrop spectrophotometer. Samples were then diluted as needed to a concentration of 10-20 ng/µl of DNA.

3.2.3 *Puccinia* specific PCR

Two *Puccinia* specific PCR primers, PuncF 5'ACCCCTAACACTTGTTTGTG-3' and PuncR 5'-GCACTAAAGGTATTGGCAAG-3', were used to determine the presence of *P. punctiformis* (Berner et al., 2015). Reactions were undertaken in 20 µl volumes, with each tube containing 10-20ng of DNA template, 10 µl of DreamTaq Green PCR Master Mix (ThermoFisher), 1 µM of each primer and the remaining volume H_2O. The cycle parameters were, an initial denaturation of 94°C for 2 min, 30 cycles, denaturing for 30 s at 94°C, annealing at 62°C for 3 min and extension at 72°C for 2 min and a final extension of 10 min at 72°C (Berner et al., 2015). PCR products were separated on a 1% agarose with a 1kb plus ladder (Thermo Scientific) used to estimate the product size. Gels were visualised after staining with ethidium bromide (0.5µg/mL), then rinsed in water and subsequently exposed to UV light. Bands present with a base pair of approximately 460 confirmed the presence of *Puccinia*.

Eleven samples from five populations in New Zealand (AppendixA.1) were sequenced to confirm the PCR results and to determine if there was any genetic difference between isolates. Sequences were analysed with the software MEGA 7. The reverse sequence was reverse complemented and aligned with the forward sequence to form a contig and compared against the other samples. A dendrogram was produced using the statistical method Neighbour joining with 1000 replications of bootstrap. A BLASTn was conducted on the samples and the sequences were compared to those on the NCBI database.

3.2.3.1 Detection threshold

The level of detection of pure *P. punctiformis* spores using the *Puccinia* specific primers (PuncF and PuncR) was investigated. DNA of the extracted spore material (spores only) were diluted in the range from 65 ng to 27pg. Spores (urediniospores and teliospores) were also directly placed into the PCR tubes with no prior DNA extraction ranging from two spores to 100 spores. The PCR reaction and cycling parameters were as described in section 3.2.3 with varying amounts of DNA template or spores.

3.2.4 Restriction Fragment Length Polymorphism (RFLP)

PCR products from 3.2.3 were digested by restriction fragment length polymorphism (RFLP). Anza Starter pack (5 enzymes) was used (ThermoFisher, 2015). All enzymes were run on at least 10 samples to see if the Punc PCR products were digested to determine if there were any genetic differences in the ITS region. The following enzymes were used: NotI (recognition site GC^GGCCGC), BamHI (G^GATCC), EcoRI (G^AATTC), XbaI (T^CTAGA) and HindIII (A^AGCTT). Reactions volumes were 10µl and contained: 1µl of 10x buffer, 1U of enzyme, 5 µl of PCR product and the remaining volume made up with H_20. Set-up of the reaction was completed on ice and then placed in a water bath at 37°C for 3 hours. Products were visualised as described in section 3.2.3.

3.2.5 Random Amplified Polymorphic DNA (RAPD) Operon Primers Kit M

Chelex DNA extraction with pure *P. punctiformis* samples (spores only) were used in random amplified polymorphic DNA (RAPD) PCR reaction. In total 37 samples were run in a RAPD PCR with samples extracted from spores only (AppendixA.1). Operon Primer Kit M (20 primers in total; Appendix A.2) were initially screened against one DNA sample and the primers producing the greatest number of polymorphisms were run against all 37 pure spore DNA samples. Three primers, OPM 6, OPM 10 and OPM 12, were used for larger population screenings. For all primers the reactions were undertaken in 25 µl volumes containing: 2.5 µl 10x buffer, 2.5mM MgCl, 200µM dNTP, 1U Platinum Taq (Invitrogen), 1 µM of primer, 10-20ng of DNA and the remaining volume H_2O. The PCR cycle consisted of an initial denaturation of 10 min at 94°C, and then 45 cycles of 1 min at

94°C denaturing and 1 min annealing at 34°C with an extension of 2 min at 72°C. The final extension 72°C for 2 min. PCR products were separated by 1.5% agarose gel and visualised as described in 3.2.3 with 5µl of 100bp DNA ladder and 1kb Plus DNA ladder. RAPD-PCR reactions were replicated three times.

3.2.5.1 Banding pattern analysis

In total 37 samples were run through RAPD PCR; not all were analysed because some samples were not reproducible. Bands needed to be present in all three replications to be analysed. Bands were analysed for each RAPD-PCR replication using Phoretix 1D pro software. The banding pattern of each individual sample was compared to one another producing a dendrogram representing matching banding patterns. The Single linkage algorithm was used to create the dendrogram with the band difference (band sharing) as the similarity coefficient.

3.3 Results

3.3.1 *Puccinia punctiformis* Polymerase Chain Reaction (PCR) ITS (internal transcribed spacer) region

Bands where produced at 460bp confirming the presence of *Puccinia* (Figure 3.1). Most samples produced a bright band irrespective of the extraction material, however samples extracted from *P. punctiformis* infected plant material produced less intense bands or no bands. A260/280 values ranged from 1.1-1.8, 1.8 being relatively pure DNA. Some samples had a faint second band. Detection of *P. punctiformis* DNA of spore material could be amplified at concentrations as low as 0.09ng/µl which produced a faint band (Figure 3.2). No detection occurred at 0.05 ng (50 pg) or 0.027 (27 pg) (Figure 3.2). Direct amplification of the spores was successful and two spores (urediniospores) were detectable in the PCR reaction (Figure 3.3). The higher number of spores (9 spores) was detected, however it is unknown whether they were only urediniospores or a combination with teliospores.

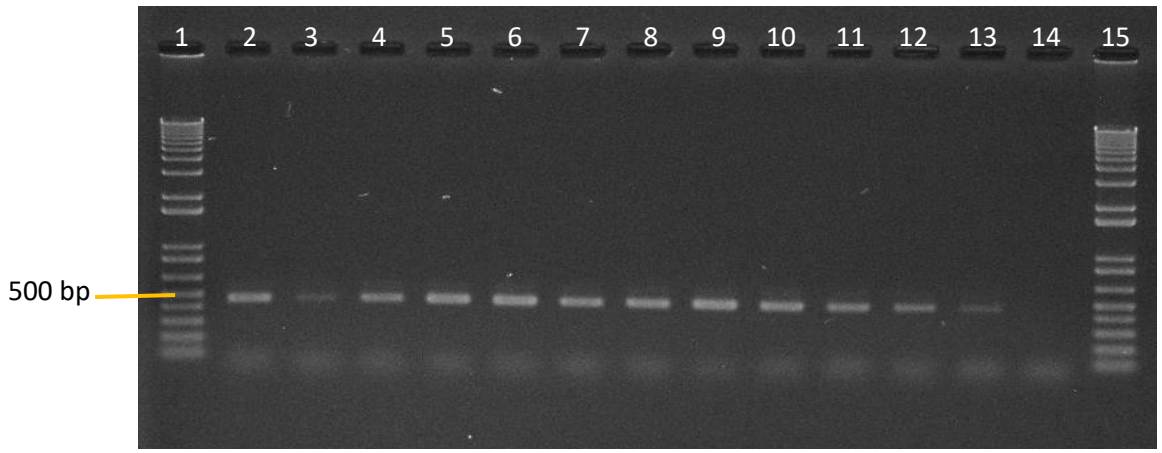

Figure 3.1: 1% agarose gel *Puccinia* specific primers in the ITS region. Bands show the presence of *Puccinia*. Lanes 1 and 15 1 kb+ ladder, lane 14 negative control. Lanes 2-13, sites (88) Highlands 2 (Bay of Plenty), (47) Hurst, (48) Hurst (Canterbury), (14) Manutuke, (18) Manutuke (Gisborne), (174) Wai –iti (Hawkes Bay), (117) Duncans (Manawatu-Wanganui), (53) Smith (Otago), (80) Milligans (Southland), (100) Poutu, (101) Poutu (Waikato) and (143) Bushfield (Wellington) respectively (Appendix A.1). These samples were also sequenced.

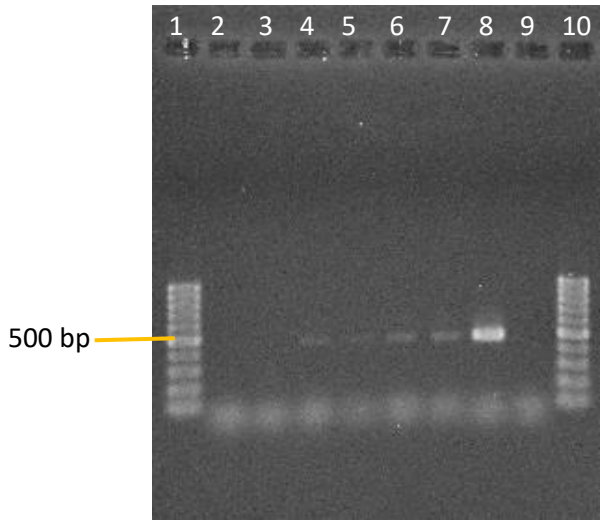

Figure 3.2: 1% agarose gel bands produced from Puccinia specific primers in the ITS region for detectable pure spore DNA levels. Lanes 1 and 10, 100bp DNA ladder, lane 9 negative control (no DNA). Lanes 2-8 increasing DNA concentrations (ng/µl) left to right: 0.027, 0.05, 0.09, 0.13, 0.18, 0.27 and 0.54ng/µl respectively.

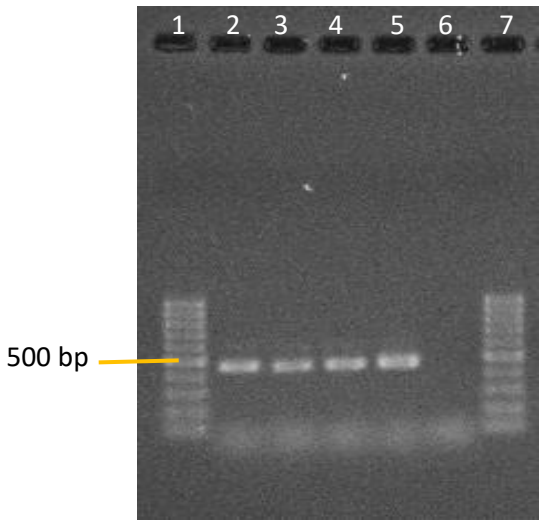

Figure 3.3: 1% agarose gel bands produced from *Puccinia* specific primers in the ITS region for detectable levels of *Puccinia punctiformis* spores in PCR tubes. Lanes 1 and 7, 100 bp DNA ladder, lane 6 negative control, lane 2, 4 spores, lane 3, 9 spores and lane 4, 2 spores and lane 5, positive control.

3.3.2 *P. punctiformis* Random Fragment Length Polymerase (RFLP), enzyme digestion

One out of five enzymes used in RFLP digested the PCR product (EcoR1). In total 20 samples from 10 populations were digested with EcoR1 with no difference observed in any of the samples (Figure 3.4). Four out of five enzymes had no digestion, fragments were 460bp long (Figure 3.5) The PCR products were digested into the approximate banding sizes: 250 bp and 50bp. The smaller fragments were unable to be visualised on the gel. All bands should add up to the original product size of 460bp.

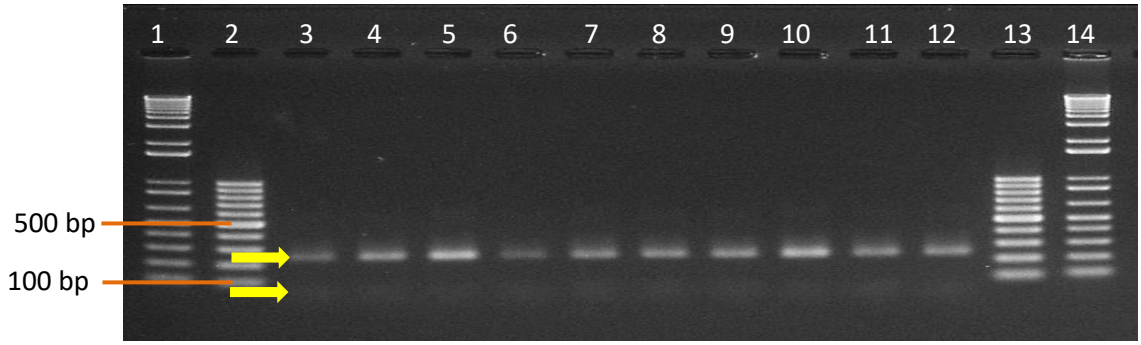

Figure 3.4: 1% agarose gel bands of RFLP digested with EcoR1 enzyme. Ten PCR products showing bands all at approximately 250bp and faint bands at 50bp (arrows), undigested PCR products are normally at 460bp. Lanes 1 and 14 1kb+ ladder, lanes 2 and 13 100bp ladder, lanes 3-12, Duncans (Manawatu-Wanganui), Manutuke (Gisborne), Manutuke (Gisborne), Clyde (Canterbury), Smith (Otago), Happy Valley (Southland), Happy Valley (Southland), Happy Valley (Southland), Milligans (Southland), Manutuke (Gisborne) (Appendix A.1).

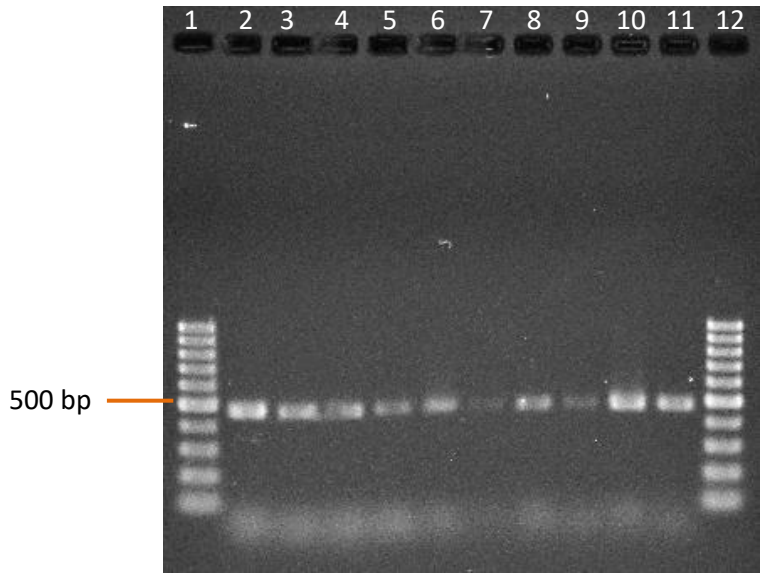

Figure 3.5: Undigested PCR product on 1% agarose gel with enzyme HindIII. Lanes 2-11 respectively, (123) Duncans (Manawatu-Wanganui), (19) Manutuke (Gisborne), (31) Manutuke (Gisborne), (40) Clyde (Canterbury), (51) Smith (Otago), (66) Happy Valley (Southland), (68) Happy Valley (Southland), (71) Happy Valley (Southland), (83) Milligans (Southland), (5) Manutuke (Gisborne) (Appendix A.1).

3.3.3 *P. punctiformis* ITS (internal transcribed spacer) region sequencing

Only one out of 11 isolates had genetic differences, the Hurst isolate (47) from Canterbury. Ten out of the 11 isolates (excluding Hurst) had not genetic differences. The Hurst isolate had a total of six nucleotide substitutions compared to the other *P. punctiformis* isolates in this study (Figure 3.6). The dendrogram (Figure 3.7) shows the different lengths of the branches, which indicates there is very little genetic differences between *P. punctiformis* isolates. The bootstrap value is 95%; all 11 isolates are very similar. The other ten samples show no genetic difference between one another. A BLAST was done on the sequences and compared against those in GenBank. On the NCBI database there were three *P. punctiformis* sequences but only one was of the ITS region and this was unverified.

Wai-iti (Hawkes Bay) Hurst (Canterbury) *P. carduorum*	AATAAACACAAATGAAATTTAAGAATGTAAAACAATTGATTAATTTTGAA AATAAACACAAATGAAATTTAAGAATGTAAAACAATTGATTAATTTTGAA AA**C**AAACACAAATGAAATTTAAGAATGTAAAACAA**A**TG**G**TTAATTTTGAA	50
Wai-iti (Hawkes Bay) Hurst (Canterbury) *P. carduorum*	ATAACTTTTAACAATGGATCTCTAGGCTCTCATATCGATGAAGAACACAG ATAACTTTTAACAA**G**GGATCTCTAGGCTCTCATATCGATGAAGAACACAG ATAACTTTTAACAATGGATCTCTAGGCTCTCATATCGATGAAGAACACAG	50
Wai-iti (Hawkes Bay) Hurst (Canterbury) *P. carduorum*	TGAAATGTGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATTGAATC TGAAATGTGATAAGTAATG**A**GAATTGCAGAATTCAGTGAATCATTGAATC TGAAATGTGATAAGTAATGTGAATTGCAGAAT**C**CAGTGAATCATTGAATC	50
Wai-iti (Hawkes Bay) Hurst (Canterbury) *P. carduorum*	TTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACACCTGTTTGA TTTGAACGC**C**CCTTGC**C**CCTTTTGGTATTCCAAAAGGTAC**C**CCTGTTTGA TTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACACCTGTTTGA	50
Wai-iti (Hawkes Bay) Hurst (Canterbury) *P. carduorum*	CTGCTATATAGCTCACTTTAAATATATAAGTCCTGTCTATGTGTGTGTCA CTGCTATATAGCTCACTTTAAATATATAAGTCCTGTCTATGTGTGTGTCA CTGCTATATAGCTCACTTTAAATATATAAGTCCTGTCTATGTGTGTGTCA	50
Wai-iti (Hawkes Bay) Hurst (Canterbury) *P. carduorum*	TGAAAATCTCTCATCAAATTAATTTTTGGTGGATGTTGAGTGCTG TGAAAATCTCTCATCAAATTAATTTTTGG**G**GGATGTTGAGTGCTG TGAAAATCTCTCATCAAATTAATTTTTGGTGGATGTTGAGTGCTG	45

Figure 3.6: DNA sequences of the ITS region two *P. punctiformis* samples and a closely related *Puccinia* species *P. carduorum*. Nucleotide substitutions are black, bold and underlined.

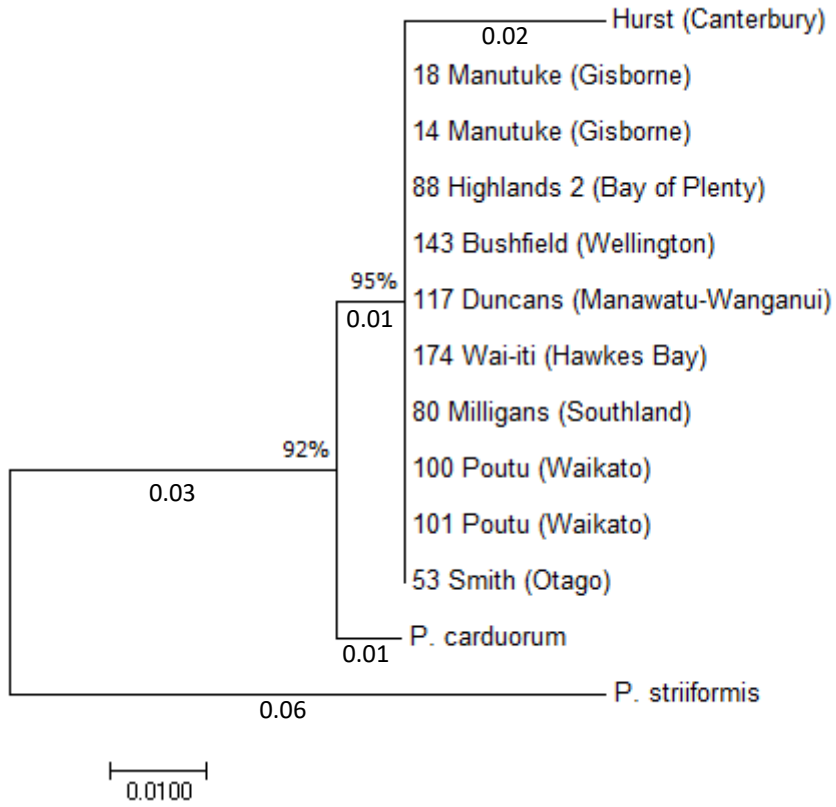

Figure 3.7: Dendrogram of sequenced samples of the ITS region with a known sequence of a closely related *Puccinia* species (*Puccinia carduorum*) and less genetically similar *Puccinia striiformis*. The site name and region are indicated. The Hurst site is genetically different from ten other *Puccinia punctiformis* isolates. Scale 1-0, 0 no diversity.

3.3.4 *P. punctiformis* Random Amplified Polymorphic DNA (RAPD)

All 20 OPM primers were screened and 10 primers (OPM 1, 4, 6, 10, 11, 12, 16, 17, 18, 20) produced polymorphic bands (Figure 3.7). The banding intensity and number of polymorphic bands differed between the 10 OPM primers. Three primers that produced the greatest number of polymorphisms were then used on pure spore samples. Some of the samples such as sample 50 from the Hurst site, only produced one band while other samples such as sample 37 from the Clyde site had up to five bands (Figure 3.8). Samples had differing banding patterns even within populations. Overall, the genetic difference in all of the samples is small as indicated by the longest branch of the dendrogram being 0.030 (Figure 3.9). There is genetic diversity both between populations and within populations. Although some populations have more samples present due to what samples were extracted from spores. Manutuke may be over-represented. Samples that were collected within the same population appear to have slight genetic differences.

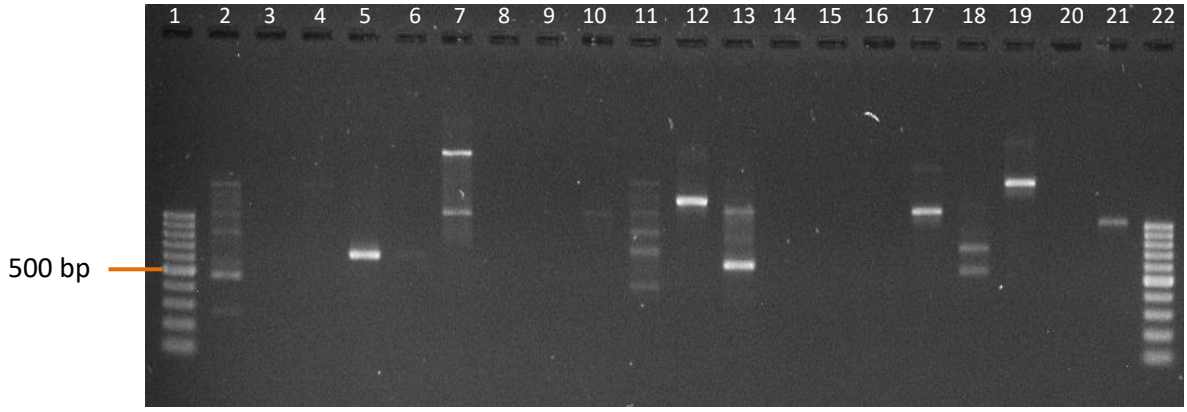

Figure 3.8: 1.5% agarose gel testing all OPM primers on a *Puccinia punctiformis* sample. Lane 1 and 22 100 bp DNA ladder. Lanes 2-21 OPM primers 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 respectively. OPM primers 1, 4, 6, 10, 11, 12, 16, 17, 18, 20 show banding patterns.

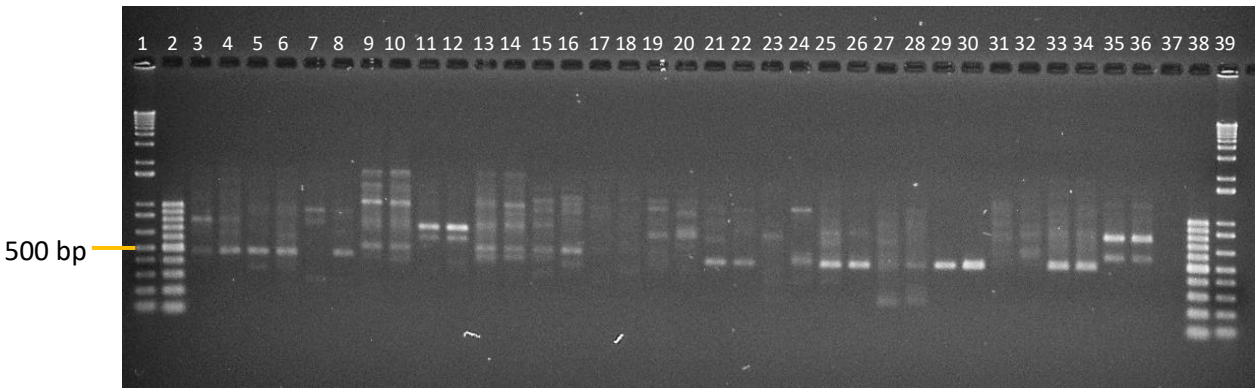

Figure 3.9: 1.5% agarose gel of RAPD OPM 12 for *Puccinia punctiformis* spore samples with replications two and three. Not all samples are shown. Lane 1 and 39 1kb+ DNA ladder, lanes 2 and 38 100bp DNA ladder, lane 37 negative control. Lanes 3-36, sample (19) Manutuke Replication 2, Replication 3, (31) Manutuke R2, R3, (36) Clyde R2, R3, (37) Clyde R2, R3, (38) Clyde R2, R3, (39) Clyde R2, R3, (40) Clyde R2, R3, (41) Clyde R2, R3, (42) McDonald R2, R3, (43) McDonald R2, R3, (44) McDonald R2, R3, (45) McDonald R2, R3, (46) Hurst R2, R3, (47) Hurst R2, R3, (48) Hurst R2, R3, (49) Hurst R2, R3, (50) Hurst R2, R3 respectively (Appendix A.1).

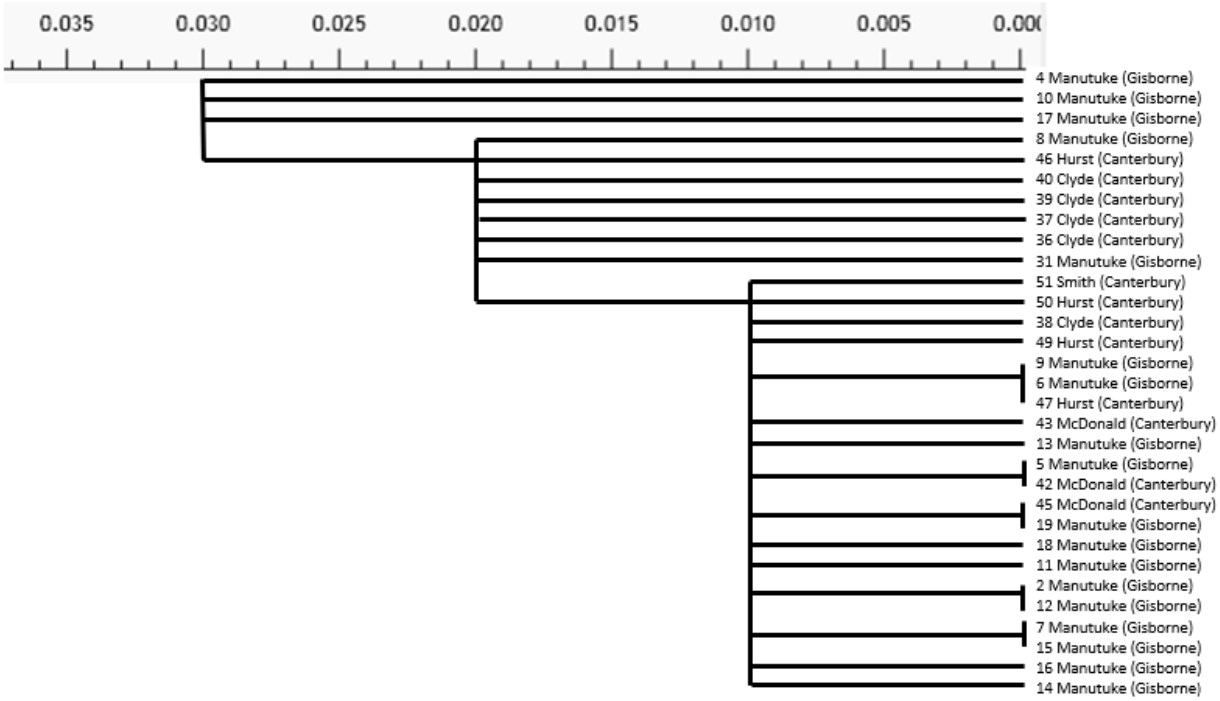

Figure 3.10: Dendrogram of RAPD OPM 12 pure spore samples. Single linkage and band differences. The top scale shows the band length which represents the amount of change between samples.

3.4 Discussion

The various analytical techniques showed there is genetic diversity in *P. punctiformis* in New Zealand, both in the ITS region and the whole genome, as indicated by sequencing and RAPD polymorphisms. The ITS region showed there was one isolate (Hurst) that was genetically different to the other ten isolates sequenced. The RAPD analysis indicated genetic diversity within and between populations of *P. punctiformis* isolates. There appears to be greater diversity between populations however there appears to be large genetic variation in the Manutuke population. The Manutuke population had more samples sequenced compared to other sites so is perhaps is not a fair representation. Genetic diversity in *P. punctiformis* may partially explain the variation in the proportion of rust infected shoots within and between *C. arvense* populations.

3.4.1 *Puccinia* specific primers and enzyme digestion

Plants collected from the survey and processed for genetic diversity were all infected with *P. punctiformis.* DNA extracted from pure *P. punctiformis* spore samples that underwent PCR, produced the brightest bands in the gels. However, not all samples after DNA extraction produced bands after undergoing PCR. This may have been a result of the degradation of plant material the rust was extracted from, and the plant phenolics may have inhibited the primers resulting in no amplification of the *Puccinia* product (De Boer et al., 1995). Some samples contained plant material thus it is difficult to determine whether the spectrophotometer reading was plant DNA or *P. punctiformis* DNA. The nanodrop gave readings for the A260/280 value indicating how pure a DNA sample is. Nanodrop values at or above 1.8 is relatively pure DNA. A260/280 readings in this experiment ranged from 1.1-1.8 indicating some samples were not pure and both *P. punctiformis* and plant DNA was most likely present (ThermoFisher/Nanodrop). The quantity of either DNA that is present is difficult to confirm using this method. The plant DNA could interfere with the PCR reaction, the primers may be unable to bind to *P. punctiformis* DNA (De Boer et al., 1995). The absence of a band could be due to poor DNA quality or the plant material present in the reaction (Schrader et al., 2012). Most likely it is the interference from the plant DNA because DNA extraction of pure spore samples were uninhibited.

The ITS-PCR protocol in this study allowed for approximately 0.09ng or 90pg of *P. punctiformis* DNA to be detected (27 urediniospores). This concentration is an approximate amount due to the nanodrop being less precise at low amounts of DNA concertation. The detectable amount of *P. punctiformis* was only tested with pure samples of rust spores, the presence of plant material could have changed the outcome. Further detection levels looked at the number of individual spores that the primers were able to detect when the spores were placed directly into the PCR reaction. Two spores were detected. This was processed with no plant material which may have changed the

number of spores that were detected. The results from this study indicate that detection of *P. punctiformis* spores *in planta* may be inhibited by plant material the plant phenolics interfering with the primers (De Boer et al., 1995). There have been other studies where fungal spores have been placed directly into the PCR reaction (Aufauvre-Brown et al., 1993; Shyamala & Ames, 1989; Yanagihara et al., 2011). The initial denaturation bursts the cells, opening and releasing the DNA, it has been found that sometimes the cell debris may interfere with the protocol (Aufauvre-Brown et al., 1993). This has not occurred in this study and indicates direct PCR of spores, without extraction, can be used.

Amplification of the ITS region is used to indicate differences between species but can be used to indicate intra-species variation, particularly for geographic races of isolates (Rasmussen, 2012b). An RFLP can be used on the PCR products produced to determine if there were any variations in *P. punctiformis* isolates. In this study, the ITS-PCR products run through RFLP indicated no genetic differences when visualised on a gel. Five enzymes were screened and only one digested the PCR product, EcoR1. The digestion produced two distinctive bands at 250 bp and 50 bp. Due to the visualisation process of the banding pattern on the gel, smaller fragments could not be seen. Other papers have found it difficult to detect fragments smaller than 20bp (Poly et al., 2001). A higher quality agarose may have visualised the remaining digested fragments. The use of enzymes that were 4 base cutters may have given different results. Four base pair cutters are better for smaller products.

3.4.2 ITS Sequence and RAPDs

Sequencing of the ITS region indicated that only the Hurst isolate genetically differed from the other ten isolates screened in this study. The 10 other isolates (excluding Hurst) had no genetic variation between one another. The difference in the Hurst isolate had variations in base pairs in the ITS1 and ITS2 region; there were 6 nucleotide substitutions. Detecting a difference in the ITS region shows that there is a genetic difference between samples as the ITS region is a relatively conserved region. The ITS region generally indicates differences between species and can be used to show geographic races (Rasmussen, 2012b). RAPDs confirmed there were genetic differences along the whole genome within and between populations. This study indicates that there is potentially more than one race of *P. punctiformis* in New Zealand but the virulence of these potential races needs to be confirmed. Greater genetic diversity in *P. punctiformis* could result in greater virulence which could explain why there are various levels of rust infection in *C. arvense* populations.

Sequencing of ITS regions showed there is genetic diversity between the Hurst isolate compared to the other ten isolates. RAPDs show there is genetic diversity within and between locations. There could be several reasons as to why isolates become genetically diverse. The different isolates could

have been introduced to New Zealand at different times. There are a lack of studies into the genetic diversity of *P. punctiformis* available, therefore may make it difficult to determine if there are genetic differences internationally, as well in New Zealand. The sequenced samples showed only one isolate that appeared to have genetic variation, the site Hurst's location is between or near the other isolate populations and thus it appears that the Hurst isolate has been genetically separated from the other isolates. RAPD results had genetic variation within and between populations, there does not seem to be a pattern as to where the different isolates are located so it is difficult to determine what areas they originated from. Isolates could have possibly been introduced separately to the area. Sampling areas that are close to the Hurst isolate in all directions would give an indication as to how far this isolate has spread and perhaps what direction it has come from.

Another reason for genetic diversity between isolates is through genetic mutations. RAPDs analyse the whole genome, therefore there could be slight difference along the whole genome as there are more alleles where mutations could occur compared to the small section of the ITS region (Gil-Lamaignere et al., 2003). Another reason as to why there could be a genetic difference is sexual reproduction and virulence adaptation, where the pathogen adapts to better infect the host (Masri et al., 2015; Thrall et al., 2002). *C. arvense* is a highly genetically diverse plant which may result in varying levels of resistance between the different genotypes. This difference in resistance could influence the genetic diversity among the pathogen. The pathogen evolves and changes to increase the level of virulence which could also involve sexual reproduction. This evolution or co-evolution relates back to the host-pathogen interaction (Masri et al., 2015; Thrall et al., 2002). When the pathogen lands on the plant, the plant may not show resistance to the pathogen and is infected. However, the plant could then develop and recognise the rust presence, and produce a response that will stop the pathogen infecting such as the hypersensitive response. The pathogen could then develop virulence to be able to infect the plant again, this co-evolution can continue (Masri et al., 2015; Thrall et al., 2002). When the pathogen develops virulence, this could change the genetics of the pathogen and therefore develop genetic diversity. Genetic diversity development in *P. punctiformis* could be due to the genetic diversity of *C. arvense*. It has been found in other *Puccinia* species such as *P. graminis* where sexual reproduction contributes to genotypic variation within a population (Berlin et al., 2014). The genetic diversity between isolates has been seen in the ITS region therefore further studies could look at different gene regions such as beta-tubulin and alpha-elongation, to determine if there is any genetic differences in these other areas and not just the ITS region (Raja et al., 2017).

Other species of *Puccinia* have genetic differences including, *P. striiformis*, *P. melanocephala, P. graminis* and other rust species including, *Phragmidium violaceum.* Therefore it could be expected that there is a genetic difference between *P. punctiformis* isolates (Kolmer et al., 2012; Kolmer &

Ordoñez, 2007). The sequencing of the ITS region and RAPDs, in this study, did confirm genetic diversity in *P. punctiformis* isolates in New Zealand. The ramifications of this diversity are the potential differences in virulence of the pathogen on genotypes of *C. arvense.*

3.4.3 Limitations and advantages

DNA extraction could be a limiting factor in the success of PCR amplification. The first limitation of this experiment could be the spore removal from plants that were collected during the survey in Chapter 2. *P. punctiformis* is biotrophic thus it is unable to be cultured *in vitro* on agar plates and there are difficulties in bulking this pathogen for extraction purposes whereby pure spore isolates are required. For this study, the spore samples were collected by brushing or flicking the spores off the plants. However, some of the plants were too dry for brush spore removal and therefore were not used in the RAPD process. RAPDs can be a difficult molecular technique to conduct but can be good to observe genetic diversity among samples. It is fast and cost efficient but lacks reproducibility. The lack of reproducibility that can be seen with the RAPD method can be attributed by the protocol conditions, the variability in DNA and the type/concentration of primers and DNA polymerase used (Hata, 2010). The RAPD technique is extremely laboratory dependent and the quality and concentration of the template DNA, as well as the cycling parameters, can greatly influence the outcome of the RAPD PCR (Tabit, 2015). Due to its poor reproducibility, Variations between runs can be separated by performing several RAPDs and electing the bands that are present in all replications. It can be difficult to analyse the banding patterns of some samples as only one band was present in all replications even if there are other bands in the other replications.

DNA extraction occurred on plant material as well as pure spores. The presence of plant material may have inhibited the primers in the PCR reaction. Berner et al. (2015) to assess the Punc primers, amplified *P. punctiformis* extracted from fresh plant material. Therefore, plant material that was used in this study may have been too old, and hence affected the quality of DNA extracted from the rust *in planta*. The primers only amplified *P. punctiformis* as this is the only *Puccinia* species that infects *C. arvense* (Berner et al., 2015; Hayes, 2005). The DNA extraction method varied from that of Berner et al. (2015) paper which could explain why the plant material may inhibit the PCR protocol. Chelex DNA extraction method was used in this study (section 3.2) whereas DNEasy Plant Mini kit (QIAGEN) was used by Berner et al. (2015), which may have been more effective at producing higher quality DNA. The different types of spores were not separated prior to DNA extraction. The different types of spores are haploid (urediniospores) and diploid (teliospores) which may explain as to why sometimes there was a second faint band. Further molecular work could investigate the effect of plant material on the primers as well as separating the different spore types prior to DNA extraction.

The products from the ITS-PCR were then used for RFLP, this method only looked at the section of DNA that was amplified during the previous PCR, in this case, the ITS region. The quality of the PCR product (the brightness of the band) could affect the quality of the resulting RFLP bands. A type of gel such as acrylamide gel or a higher molecular grade agarose instead of the low-grade agarose because not all digested bands were visible. RFLP are techniques that are known to have the most reproducible results, it is also fast and simple (Tabit, 2015). Different gels could be used such as acrylamide gel or a higher molecular grade agarose to better visualise the smaller bp bands.

3.4.4 Further studies

If further studies of *P. punctiformis* are undertaken, the use of pure spores is needed, this could be accomplished by directly extracting the spores off the plant in the field with a suction tool. Other molecular techniques that could be used include amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) (Karp et al., 1996). Different DNA extraction methods could be used such as DNEasy used in Berner et al. (2015), where plant material appeared to have no effect on the amplification of the primers. The use of higher quality agarose will aid in better banding pattern visualisations especially with techniques such as RFLP. If cost is not a limitation then sequencing and undertaking more molecular work on more samples will give a better idea on the genetic difference of *P. punctiformis* across the country and perhaps give a map indicating locations of the different isolates. Identifying the genotype or looking at the genetics of the thistle and connecting it with the genetics of the pathogen may aid in understanding the relationship and the interaction of the host and pathogen. Instead of looking at the whole genome where genetic differences are more likely other gene regions along with the ITS region could be looked more closely at, these could include β-tubulin or alpha-elongation.

3.5 Conclusion

P. punctiformis samples were collected around New Zealand and underwent several different molecular techniques to determine whether there was a genetic difference between samples. The results showed that there was genetic diversity among samples in both the ITS region, where one isolate was genetically different to the other ten isolates. Within the whole genome, where there was more genetic diversity between isolates and even between populations and within regions. Further studies could look at the combined genetic diversity of the plant and the pathogen as well as looking at the distribution of the different isolates.

Chapter 4

Puccinia punctiformis in planta

4.1 Introduction

Puccinia punctiformis is a highly host specific rust fungus that only infects *Cirsium arvense. C. arvense* is an invasive weed in New Zealand and temperate regions of the world. *Puccinia punctiformis,* can systemically infect *C. arvense* and move into the roots. It is not completely understood whether the fungus directly kills the roots, however, it is known to overwinter in the roots (Berner et al., 2013). In spring some shoots emerge that are systemically infected which results in the pathogen killing the shoots. This subsequently reduces root biomass and potentially diminishes thistle shoot abundance (Berner et al., 2013; French & Lightfield, 1990).

Many pathogens can systemically infect their host and several studies have looked at how they move within the plant. *Puccinia thlaspeos* grows down the leaves of its host and into the petiole moving into new leaves of the plant. The pathogen is thought to infect the leaves and then move through the meristematic area, where it overwinters (Kropp et al., 1996). Pathogens can also move through the phloem, depending on the pathogen and other vectors such as insects and where they penetrate the plant (Zhu et al., 2002). When fungal pathogens infect a host they produce specialised infection structures, such as an appressorium which aids in penetration of the host (Allen et al., 1991; Hoch & Staples, 1987). The position of the appressorium is important for most rust fungi because it needs to develop over the stomatal opening for successful infection (Allen et al., 1991; Hoch & Staples, 1987). *Puccinia carduorum,* a rust fungus of a different thistle (*Carduus tenuiflorus*), can detect the height of the ridges of the leaf for appressorium development, with the optimal height being approximately 0.4-0.8µm (Allen et al., 1991). Heights, higher or lower than this range, decreases appressorium development resulting in decreased infection (Allen et al., 1991; Hoch & Staples, 1987). A study observed the distribution of two different autoecious rust fungi in the vascular system once penetration occurred. Autoecious fungi complete their life cycle on one host. Of the five spores types that can possibly be formed in a macrocyclic rust fungus, a large percentage of the spores found within the plant were urediniospores and aeciospores (Baka & Lösel, 1992a).

The quantification of DNA is a useful research technique as it indicates how much of a pathogen is in a host and where it is concentrated (Dworkin et al., 2002; Peirson & Butler, 2007). Quantitative polymerase chain reaction (qPCR) or real-time PCR is a molecular technique based on the Polymerase Chain Reaction (PCR). Quantitative PCR can be used to quantify the amount of pathogen using specific primers. When the DNA is amplified by the primers it is monitored at each cycle. The

different levels of amplified DNA are detected by a fluorescent dye most commonly SYBR Green (Arya et al., 2005). The PCR cycle at which the fluorescence of the samples crosses the threshold is the threshold cycle or Ct. Therefore, the higher concentration of DNA the more fluorescence and the earlier it crosses the threshold (Heid et al., 1996; Ramakers et al., 2003). Understanding how *P. punctiformis* moves *in planta* and determining where the pathogen is concentrated will give a better understanding of the host-pathogen interaction especially throughout both lifecycles and throughout the seasons.

The aim of this body of work is to determine, at a giving point of time the level of infection *in planta* caused by *P. punctiformis*. This will give an indication of where the greatest concentration of spores is located as there is little literature with regards to *P. punctiformis in planta*.

4.2 Material and Methods

4.1.1 DNA extraction Chelex

In total nine plants were collected from the Clyde site, near Springston Canterbury (longitude 172.40076, latitude -43.679973 and with an altitude of 10m), where *P. punctiformis* was present. Six plants visibly infected with *P. punctiformis* pustules (plants 1, 2, 4, 7, 8, and 9) and three plants not visibly infected with *P. punctiformis* pustules (plants 3, 5 and 6) were collected. The plants were stored in a -20°C freezer until processing. To process the plants, 1cm sections were cut which included the top (S1), middle (S2) and bottom (S3) of the main shoot. Three leaves were taken from each plant, the top, middle and bottom. Each leaf was cut into three sections 1-3, and labelled as the tip (1), middle (2) and bottom (3) with each section cut into 10 mm x 20 mm pieces (Figure 4.1). In total 12 sections were taken from each plant with three replications. The sections of the plant were sterilised in 10% bleach for 30 s, then 2 x 30 s in H_2O, to remove any external sources of rust. DNA was extracted using a 10% Chelex 100 (BioRad) solution. For each plant DNA was extracted from 12 sections; in total 80 sections were processed. The plant material (10 mm x 20 mm) was ground in liquid nitrogen using a mortar and pestle, then placed into 1.7 ml tube containing 300µl of 10% Chelex solution. Tubes were vortexed for 10 s three times and then placed into a heating block at 100°C for 10 min. The tubes were removed from the heating block and vortexed and returned to the heating block for a further 10 min at 100°C. Tubes were then centrifuged for 10 min at 13,000g. The supernatant (approximately 150 µl) was removed and placed into 0.6 ml tubes. The DNA concentration was measured using a Nanodrop spectrophotometer. Samples were diluted with sterile Millipore H_2O to 30-50 ng/µl.

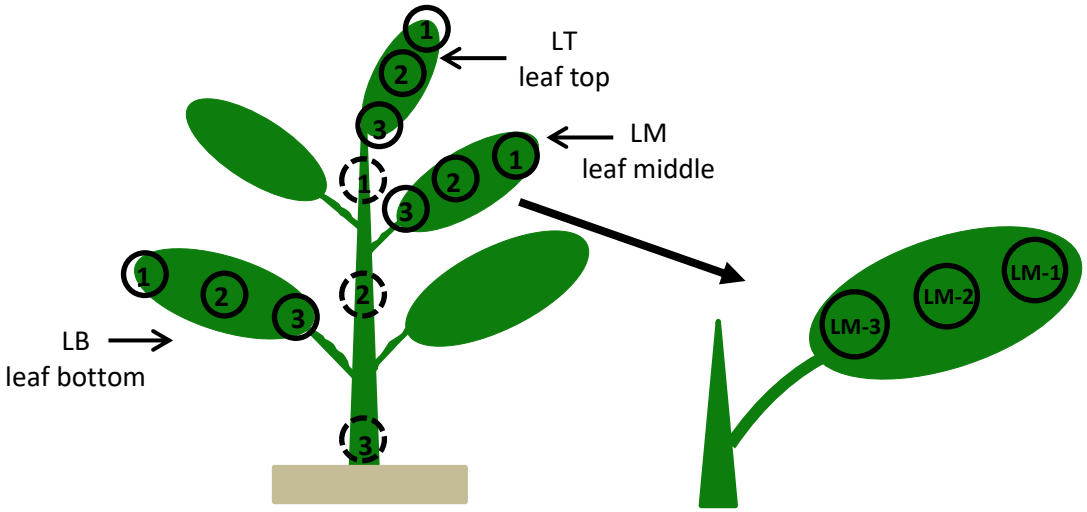

Figure 4.1: Sections of the plant for qPCR. Solid circles (○) leaf samples, dashed circle (◌), shoot samples. Numbers 1-3 indicate the position of the samples on the shoot or leaf, 1, tip of the leaf, 2 middle of leaf and 3 end of the leaf closest to stem. For the shoot 1 top of leaf, 2 middle, 3 bottom. For each plant 12 sections in total, 3 for each leaf and 3 for the shoot.

4.2.2 qPCR

All qPCR cycles were undertaken in a Rotor-Gene Q (QIAGEN) machine. Reactions were undertaken in 10 µl volumes. Each tube contained 10-20 ng DNA, 5 µl SYBR green (QIAGEN), 0.13 µM of forward primer PuncF (5' ACCCCTAACACTTGTTTGTG 3') and reverse primers, PuncR (5' GCACTAAAGGTATTGGCAAG 3') (Berner et al., 2015), with the volume made up to 10µl with H_2O. Four standards, of the rust fungus were used with a known concentration 1.1ng, 11ng, 36.25ng and 72.5ng, there were two negative standards, one with water and one with *C. arvense* DNA containing no rust. The rust samples were taken from the Clyde site and the DNA was extracted with the method from 4.1.1. The qPCR cycle is as follows, the first cycle, denaturing at 94 °C for 10 min, then 35 cycles, denaturing 94 °C for 10 s, annealing 58 °C for 20 s and extension 72 °C for 30 s and 1 cycle of final extension 72 °C for 2 min. Any positive detection after 30 cycles were run through gel electrophoresis on a 1% agarose for 45 min at 90v and stained in ethidium bromide for 30 min, rinsed with water for 15min and then visualised under UV. qPCR reactions were undertaken three times with a total of 240 reactions undertaken. Concentration (ng/µl) results were analysed in Minitab. The data was analysed using an ANOVA generalised linear model and Sidak pairwise comparison, prior to analysis, data was transformed with ln(x+1) to obtain normality. The number of spores per microlitre were calculated where the average genome size of Pucciniales is 305.5 Mbp and 1pg is 978 Mbp. Therefore 1 nuclei of Pucciniales is equivalent to roughly 0.3pg (Tavares et al., 2014). Calculations were made assuming spores are 95 % urediniospores which has 1 nuclei and 5% teliospores with 2 nuclei.

4.3 Results

4.2.3 *Puccinia punctiformis* concentration (ng/µl) *in planta* in all thistles.

All *C. arvense* collected had detectable amounts of *P. punctiformis* ranging from 0.1-5000 (ng/µl). The average *P. punctiformis* concentration differed significantly (P<0.001) between locations within the nine plants (Figure 4.2). The trend indicates that the top and middle leaves on the plant have higher concentrations of *P. punctiformis* and the concentration decreases down the plant, towards the base (Figure 4.3). The interaction between location and sub-location within all plants was significantly different (P<0.05), LT1 (leaf top, tip) had the highest concentration 1490 ng/µl while LB2 (leaf bottom, middle) had the lowest concentration 51 ng/µl (Table 4.1). It shows that the S (shoots) and LB have the lowest amount of *P. punctiformis* compared to the other locations. There appears to be no trend between sublocations.

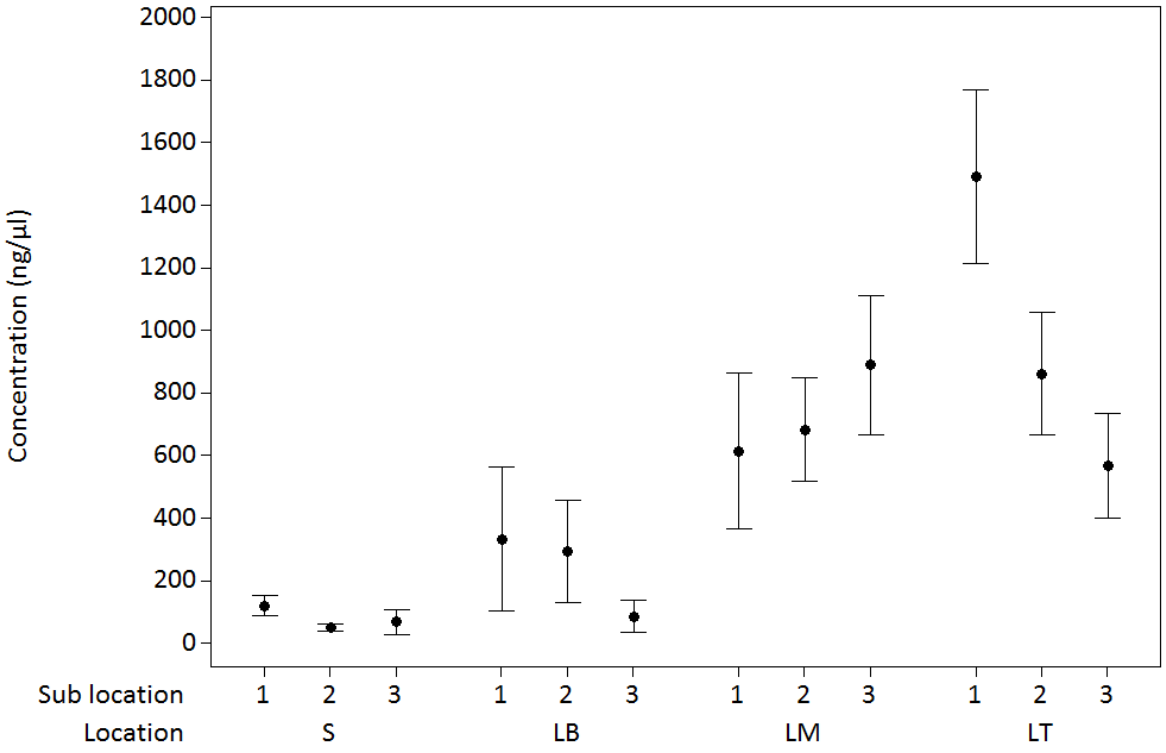

Figure 4.2: Average *Puccinia punctiformis* concentration (ng/µl) *in planta* across nine thistles collected from infected Clyde site, Canterbury, with standard error of mean (SEM). Locations, LB, bottom leaf, LM, middle leaf, LT, top leaf. Sublocations 1-3 are sections within the location, 1, tip, 2, middle and 3, bottom section closet to stem.

Table 4.1: Average *P. punctiformis* concentration (ng/µl) at different locations and sub locations across nine thistles collected from a site infected with *P. punctiformis* in Canterbury. Grouping was determined by statistical analysis using transformed data and a Sidak comparison. Spores/µl assuming all spores are urediniospores, 1 nuclei.

Location	Original data (ng/µl)	SE Mean	Spores/µl (Approximately)	Grouping
LT 1	1490	± 278	4.36×10^5	A
LT 2	862	± 195	2.52×10^5	A B
LM 3	889	± 223	2.60×10^5	B
LM 2	683	± 861	2.00×10^5	B C
LT 3	568	± 169	1.66×10^5	B C
LM 1	615	± 250	1.80×10^5	C D
S 1	121.3	± 32.8	3.55×10^4	D E
LB 3	86.4	± 50.8	2.02×10^4	E F
S 3	69.1	± 39.1	1.49×10^4	E F G
S 2	51	± 12.2	1.49×10^4	E F G
LB 1	333	± 229	9.74×10^4	G
LB 2	296	± 163	8.66×10^4	G

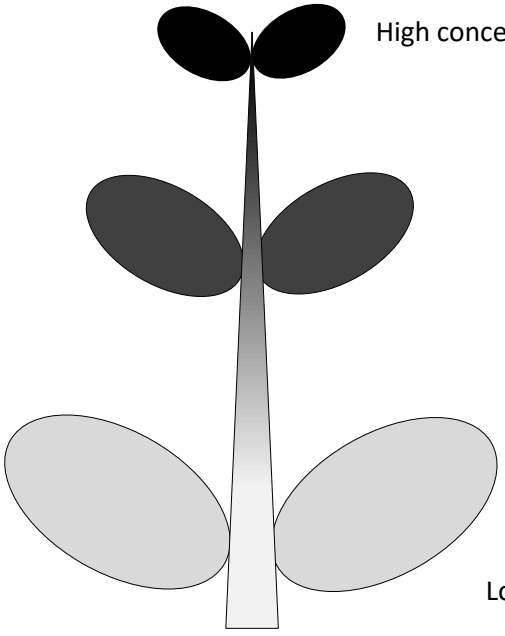

Figure 4.3: Plant gradient, *Puccinia punctiformis* concentration decreases down the plant. Highest concentration at the top leaves, lowest at the bottom leaves. Lower concentrations in the shoot. The darker the colour, the higher the concentration of *Puccinia punctiformis.*

4.3.3 *Puccinia punctiformis* concentration *in planta* in individual thistles

All *C. arvense* collected had varying levels of detectable amounts of *P. punctiformis* ranging from 0.1-5000 (ng/µl) (Figure 4.4). There was a significant difference (P<0.001) between the nine plants where DNA was detected (Figure 4.4, Table 4.2). The six plants with visible *P. punctiformis* pustules had

higher levels of *P. punctiformis* (230-1300 ng/µl on average) compared to asymptomatic plants which had extremely low levels of *P. punctiformis,* below 16 ng/µl (Table 4.2). Plant nine had the highest amount of *P. punctiformis* (1272ng/µl) while plant three had the lowest (<1ng/µl). There was a significant plant x location interaction (P<0.001). For each plant, the location with the highest concentration of *P. punctiformis* varied (Appendix B.1). Plant 2 (Figure 4.5) and Plant 7 (Figure 4.6) from the Clyde population, differed in locations within the plant in regard to the highest concentrations of rust. In Plant 2 (Figure 4.5) the top leaf (LT) (average 851.7 ng/µl) had the highest concentration compared to the other locations, whereas plant 7 (Figure 4.6) the middle leaf (LM), had the highest concentrations (average 1031.7 ng/µl) compared to the other locations within the plant. There were no significant (P>0.05) differences in the quantity of rust within a sublocation for seven out of the nine plants. Plants 1 and 8 had a significant difference between sublocations (P<0.02). There was a significant (P<0.001) interaction between location and sublocation for seven out of the nine plants. For example in plant 2 there is a significant difference (P<0.05) between LT1 and LM1. However, there was no significant difference for this interaction in plants 1 and 5 (P>0.05).

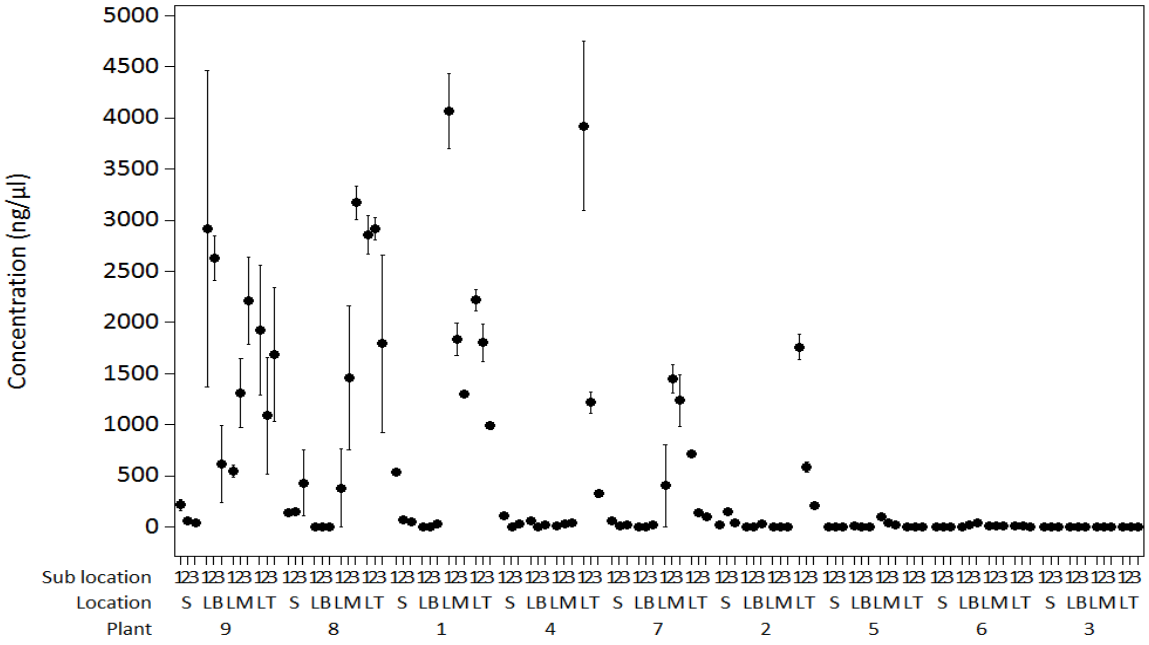

Figure 4.4: *Puccinia punctiformis* concentration (ng/µl) *in planta* in six thistles visibly infected with *P. punctiformis* (1, 2, 4, 7, 8 and 9) and three thistles not visibly infected with *Puccinia punctiformis* (3, 5 and 6). Collected from Clyde site in Canterbury where *Puccinia punctiformis* was present. With standard error of mean (SEM). Locations, LB, bottom leaf, LM, middle leaf, LT, top leaf. Sub locations 1-3, 1, tip, 2, middle and 3, base of leaf/shoot.

Table 4.2: *Puccinia punctiformis* mean concentration (ng/µl) in nine *Cirsium arvense* thistles from a *P. punctiformis* infected site in Canterbury. Grouping was determined by statistical analysis using

transformed data and a Sidak comparison. Spores/µl assuming all spores are urediniospores which contain one nuclei.

Plant	Original data (ng/µl)	SE Mean	Spores/µl	Grouping
9	1271.62	± 214	3.72×10^5	A
8	1109.63	± 223	3.25×10^5	B
1	1075.43	± 204	3.15×10^5	B
4	483.28	± 192	1.41×10^5	C
7	347.83	± 89.8	1.02×10^5	C
2	234.16	± 82.9	6.85×10^4	D
5	15.10	± 5.04	4.42×10^3	E
6	10.41	± 2.73	3.04×10^3	E
3	0.89	± 0.519	2.60×10^2	F

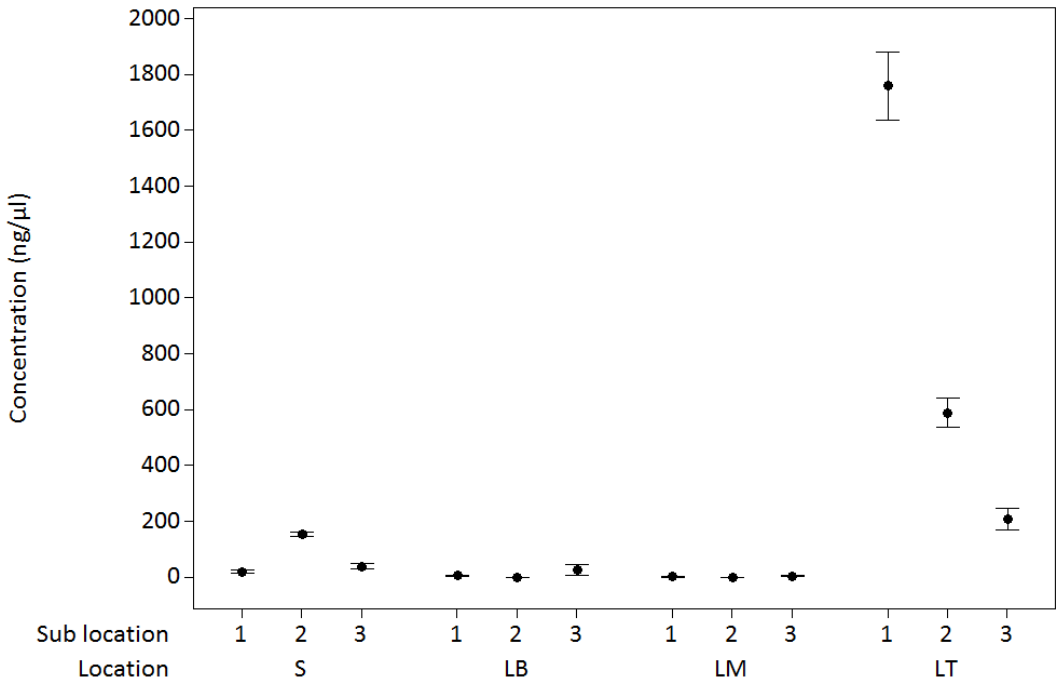

Figure 4.5: *Puccinia punctiformis* concentration (ng/µl) in Plant 2 collected from *Puccinia punctiformis* infected Clyde site, Canterbury. With standard error of mean (SEM). Locations, LB, bottom leaf, LM, middle leaf, LT, top leaf. Sublocations 1-3, 1, tip, 2, middle and 3, base of leaf/shoot.

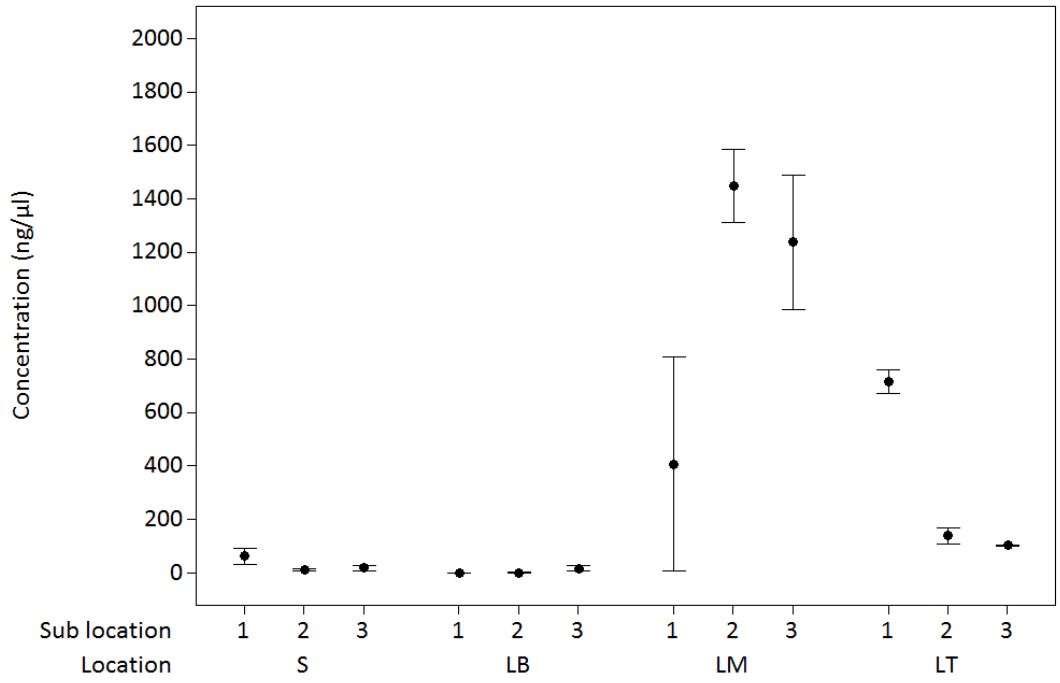

Figure 4.6: *Puccinia punctiformis* concentration (ng/µl) in plant 7, collected from *Puccinia punctiformis* infected Clyde site in Canterbury. With standard error of mean (SEM). Locations, LB, bottom leaf, LM, middle leaf, LT, top leaf. Sub locations 1-3, 1, tip, 2, middle and 3, base of leaf/shoot.

4.4 Discussion

P. punctiformis is a classical biological agent that can systemically infect *C. arvense* resulting in reduced shoot abundance (Berner et al., 2013). The concentration and movement of *P. punctiformis* within the plant is not fully understood but there have been studies into the infection of the vascular tissue by other rust fungi (Baka & Lösel, 1992a). The concentration of *P. punctiformis* was determined in this study, sampling nine plants from the same population at the end of the growing season (Autumn). This study has indicated the abundance (concentration) and location of *P. punctiformis* spores in *C. arvense* shoots. This new information will aid in understanding the host-pathogen interaction and the pathogen lifecycle.

All plants had detectable amounts of *P. punctiformis* within the plant. Results showed that the highest concentration is generally at the top of the plant and decreases down the plant. Plants that had no visible symptoms had low levels of *P. punctiformis*. Nine plants in total were collected from a site in Canterbury that had *P. punctiformis* present. Six plants with visible *P. punctiformis* pustules and three plants with no obvious visible symptoms (plants 3, 5 and 6) were sampled. The different plants had varying levels of *P. punctiformis* from less than 1 ng/µl to 5000 ng/µl in varying locations in individual plants. It was found that despite asymptomatic plants, *P. punctiformis* was present in

the vascular system in all plants sampled. Surface sterilisation of the sections occurred; this excluded the *P. punctiformis* on the outside but determined the concentration within the plant. The plants were taken at a point in time and thus there is no way to determine the stage of infection and the pathogens progress prior to sampling.

4.4.1 *Puccinia punctiformis* concentration *in planta*

The average concentration of *P. punctiformis* in nine plants at different locations within the plant, indicated that there were higher concentrations of *P. punctiformis* in the growing points of the plant, the top of the plant. *P. punctiformis* spores decreased further down the plant with the bottom leaf having the lowest concentration. The concentration of *P. punctiformis* varied in different locations in individual plants. The top leaf of Plant 2 had the highest *P. punctiformis* concentration while the middle leaf had the lowest. Compared to Plant 7 where the middle leaf had the highest concentration while the bottom leaf had the lowest concentration. The plants that showed no symptoms of *P. punctiformis* appeared to have a slight pattern as to where *P. punctiformis* was located, the middle leaf had the highest concentration group for each plant (3, 5 and 6) and the shoots was in the lowest group for each plant. However, Plant 5 which was asymptomatic, had higher amounts of rust in the bottom leaf compared to the top leaf.

The shoot, on average, had comparable concentrations of rust to the bottom leaves and lower amounts compared to the top of the plant. There are several reasons as to why the shoots had lower amounts of *P. punctiformis.* The pathogen could be using the shoot for translocation to the higher leaves where they are typically younger and potentially not as tolerant to the pathogen in comparison to the lower and older leaves. It is unknown how the pathogen moves within the plants. The two main types of transport tissues seen in plants, are the xylem and the phloem. It is more likely that the translocation occurs in xylem as this is transporting water and solutes upwards from the roots to the leaves. *P. punctiformis* overwinters in the roots and therefore may be taken up with the water and solutes (Cubero et al., 2006; Dunford, 2010; Sukno et al., 2007). Another indication as to why there were lower levels of pathogen in the shoots compared to the leaves is because when symptoms occur and develop in the plant, the shoots generally show symptoms later in the disease lifecycle compared to the leaves (Thomas et al., 1994). The shoots are more rigid than the leaves which may make it more difficult for the pathogen to penetrate the plant. The shoot is in the middle of the plant and therefore could be protected by the leaves which prevents the pathogen from reaching the shoot as the spores will land on the leaves first, when epiphytically rather than endophytically infected.

On average, the leaves had higher amounts of *P. punctiformis* compared to the shoot. The leaves are on the outside of the plant exposed to the elements and therefore have a higher chance of localised

infection. The bottom leaves had lower amounts of rust compared to the higher leaves, top leaf and middle leaf. The lower amounts of *P. punctiformis* in the bottom leaves could be contributed to the lower leaves being older and beginning to senesce. When the leaves start to senesce, the biotrophic pathogen may recognise this process and move away from the dying plant tissue towards healthy tissue (Cripps et al., 2009). In another study on *C. arvense* the effect of *Alternaria cirsinoxia* were investigated on leaf maturity, infection site and application rate on conidia infection. It was found that the leaf maturity had no effect on the germination of conidia but older leaves had higher appressorial formation. There appeared to be more of an effect on the older leaves compared to the younger leaves, including larger lesions and penetration of the older leaf (Green & Bailey, 2000). This does not appear to be the case with *P. punctiformis* infection of *C. arvense. A. cirsinoxia* is not a biotrophic pathogen whereas *P. punctiformis* is and does not like senescing leaves. The wax cuticle on leaves generally builds up over time and therefore it could be expected that the older leaves have a thicker wax layer. The thicker the wax the more difficult it could be for the spores to navigate through the ridges of the leaves to the appropriate site for appressorial development and penetration into the leaves (Buschhaus & Jetter, 2012; Hodgson, 1973).

All plants varied with different concentrations of *P. punctiformis.* Plant nine had the highest concentration while plant three had the lowest concentration. Plants within a population could be different genotypes resulting in varied morphological differences between the ecotypes of the genotypes (Hodgson, 1973; Solymosi & Nagy, 1998). No genetic work was down on the thistles so it is unknown whether the thistles were genetically the same. There are several morphological characteristics that could influence the amount of infection that occurs. Increased infection could be caused by an increased number of stomatal pores. More stomata results in a higher number of entry points for *P. punctiformis* (Moore, 1975; Ozcan et al., 2015). Different genotypes of *C. arvense* may also have different thickness of wax layer the thicker the wax the more *P. punctiformis* may be inhibited at infecting the plants. The wax layer may influence the spores' ability to navigate along the ridges of the leaf to find a stomatal pore for entry (Buschhaus & Jetter, 2012; Hodgson, 1973). The hairiness of a leaf may also have similar effects as the wax layer does. The more hair on the leaf the more difficulty the spore may have at finding a stomatal pore for penetration (Buschhaus & Jetter, 2012; Hodgson, 1973; Moore, 1975; Ozcan et al., 2015; Solymosi & Nagy, 1998). This could be investigated.

The varying levels of *P. punctiformis* in the different plants collected could be due to different stages of infection, or infection occurred at different times during the season. Either the plants was systemically infected from the previous season or infected during the sampling year. The plants life cycle and the stage it is at may also influence the amount of pathogen present. A combination of both the pathogen and plant lifecycle could have the same effect. Younger plants may have more

infection because they are less rigid, therefore easier for the pathogen to penetrate and infect. Younger leaves are smaller in size and therefore stomatal pores are closer together and perhaps make it easier for the pathogen to infect the plant as there is less distance for *P. punctiformis* to navigate to an entry point (Green & Bailey, 2000; Ozcan et al., 2015). This could explain why the tip of the leaf generally had higher levels of rust compared to the base of the leaf as well as why the top of the plant had higher concentrations of *P. punctiformis* compared to the base. The different ages of the plant may also influence the amount of wax build-up resulting in variable infection (Ozcan et al., 2015).

The opposite could be said for older plants, more infection might be seen because they were infected earlier than the younger plants. The pathogen may have had a longer time to infect the plant while the younger plants may be in the early stages of *P. punctiformis* disease lifecycle and therefore less infection (Berner et al., 2013; Johnston, 1990). The stage of the disease lifecycle may influence the amount of infection. If the pathogen has overwintered in the plant then it has already infected the plant and is waiting for the shoot to emerge. If the pathogen infects locally, during the sampling season, it needs to be able to successfully penetrate and infect the plant and therefore may be slightly delayed in the lifecycle, therefore for plants locally infected may have lower amounts of *P. punctiformis*. The asymptomatic plants could be at the beginning of the pathogens' lifecycle and has not yet expressed symptoms or the plant had defended itself from further infection by the pathogen (Kluth et al., 2001). The type of infection, localised or systemic infection, may influence the location of *P. punctiformis*. Localised infection may influence the location of the concentrated pathogen as there could be higher levels of the rust at the entry sites. It is the interaction between the host and pathogen that influences the amount of infection that will occur and therefore the amount of *P. punctiformis* systemically found in the plant. The combination of both *P. punctiformis* and *C. arvense* lifecycles may influence the amount and location of rust infection (Berner et al., 2013; Johnston, 1990). The genetic diversity within *C. arvense* populations could explain why there are varying levels of *P. punctiformis* concentration within and between sites. *C. arvense* is known to be highly genetically diverse, it is known to have a wide range of clonal differentiation which suggests that sexual reproduction occurs in populations (Bommarco et al., 2010; Hettwer & Gerowitt, 2004).

4.4.2 Limitations and future studies

With all molecular work, there are limitations. Before the qPCR protocol was finalised, different concentrations of primers were trialled to determine the best concentration. For the forward and the reverse primers concentration, 0.13µM produced the best curves with plant rust spore standard. The cross threshold in a qPCR can be affected by various parameters, such as primers and DNA quality. qPCR generally amplifies small products however the *Puccinia* specific primers, designed for PCR,

amplify a large product 460bp. The duration of denaturing, annealing and extension of the qPCR cycle may enhance the amplification of the DNA product. An increased denaturing time, annealing and extension time the cycles could aid in amplifying the large sequence (460bp) which was a limiting factor. Denaturing for a longer time period allowed for greater separation of strands of the DNA. Annealing for a longer time enabled the primers to attach to the DNA strands. The negative samples that had no rust present, had a curve that went above the threshold after 30 cycles. Anything that generally comes up after 30 cycles can be disregarded as this can be because of background noise or reduced efficiency (LifeTechnologiesCorporation, 2012). This can be checked by running the qPCR products that are positive after 30 cycles through gel electrophoresis and then visualised under UV light. When no bands are present there is no rust amplification.

Further studies and experiments can look at the infection and rust concentration at different times of the year and season which would result in looking at different stages of the life cycle of both *C. arvense* and *P. punctiformis*. Sampling plants at the beginning of the season with and without symptoms could determine which plants are already systemically infected. If they are systemically infected it is unclear whether the pathogen starts at the top and then moves down to the bottom of the leaves or it starts at the bottom and moves towards the top. Sampling the roots would be ideal to look at the systemic infection or overwintering of the pathogen, it would give clearer indication whether a plant was infected prior to emerging in the spring or whether the plant was infected after emergence. Roots were not tested due to the sampling method. This would help in understanding how *P. punctiformis* moves through the plant as sampling is done at a certain point in time. If sampling in the field could be accomplished without removing the whole plant, the movement of the spores *in planta* could be observed. However, this could be difficult as once the plant is cut this could either increase the chance of infection or influence where the pathogen is concentrated. There have been different studies looking at pathogens movement within its host and has been thought to play an important role in understanding the disease life cycle as well as the host-pathogen interaction (Baka & Lösel, 1992a; Cubero et al., 2006; Sukno et al., 2007). It may be beneficial to do genetic work on the plants to determine whether the plants are genetically different or they are from the same root stock. These studies will give a better understanding as to why some plants are infected and show symptoms while the majority of the population does not appear to be affected by the pathogen.

4.4 Conclusion

The quantitative polymerase chain reaction is a good method to determine the quantity of *P. punctiformis* in different locations within plants of *C. arvense.* This experiment has allowed the levels of rust infection in *C. arvense* in specific plant samples at a given time, to be determined. However,

the plants were sampled towards the end of the season end of March and several more experiments will need to be conducted looking at different stages of both the pathogen and the plant lifecycles. More plants should be sampled along with the roots of plants with visual symptoms and plants without symptoms. This experiment concluded that generally there is more *P. punctiformis* present at the top of the plant compared to the bottom and the shoots. Plants that showed no visible symptoms appeared to have low levels of rust within the plants and therefore are asymptomatic. Monitoring plants that are asymptomatic and sampling them throughout the plants' lifecycle may indicate whether the plant has a defence mechanism which will limit the pathogens spread and infection or whether symptoms will eventually express. The reason as to why there are different levels of *P. punctiformis* within a plant warrants further investigation throughout the season.

Chapter 5

Plant infection

5.1 Introduction

The rust fungus, *Puccinia punctiformis,* is a highly host specific pathogen that only infects the noxious weed *Cirsium arvense.* The potential classical biological control agent, *P. punctiformis,* is present in New Zealand but its effectiveness in controlling *C. arvense* is sporadic (Cripps et al., 2011; Johnston, 1990). The rust fungus, although it self-perpetuates, has not caused epidemics in *C. arvense* populations in order to reduce the competitive ability of the weed to levels below the economic threshold (Hill 1987; Johnston 1990). One of the major issues with *P. punctiformis* is the high variability of infection in *C. arvense* populations throughout New Zealand (Berner et al., 2013; French & Lightfield, 1990). In some populations of *C. arvense* infestation, there can be several thistle individuals that are infected and covered with *P. punctiformis* pustules, whilst other shoots are not. This can be observed in plants in close proximity to the infected *C. arvense* plant expressing no symptoms. Even shoots that are connected and part of the same ramet of an infected individual can have no visible signs of infection (Berner et al., 2013; French & Lightfield, 1990). Why this occurs is not fully understood. Understanding how *P. punctiformis* infects *C. arvense* is important to determine how infection occurs within the plant and how control of the weed may be achieved.

For fungal pathogens to be successful biological control agents, they need to spread, reproduce and control the weed, infecting whole populations not just a few individuals within the population (Briese & Sindel, 2000; van Driesche et al., 2008). Simulating the infection process is important in order to ascertain the progression of the infection *in planta.* Mowing has been found to increase *P. punctiformis* spread and infection, and this can be simulated by cutting the tops off the plants. It is not fully understood whether the increased infection is due to the mower spreading the *P. punctiformis* spores or giving the spores entry points through the cut shoots and leaves (Bourdôt et al., 2011; Demers et al., 2006). Spraying or painting-on the spores has been a common method for application of *P. punctiformis* urediniospores/aeciospores, this is generally done to the underside of the leaf as this is where the rust pustules form (Berner et al., 2015). On the underside of the leaves, there are higher densities of stomata for *P. punctiformis* to enter through and infect the plant this way (Berner et al., 2015; Moore, 1975). When the spores land on the leaf surface, they germinate and detect the height of the ridges of the stomata, with the optimal height being approximately 0.4-0.8µm (Hoch & Staples, 1987). An appressorium then develops over the stomatal opening enabling penetration and internal infection (Allen et al., 1991; Hoch & Staples, 1987).

Research has been undertaken to determine the optimal conditions for germination of teliospores and urediniospores of *P. punctiformis.* Thomas et al. (1994) and Watson and Keogh (1980) determined that teliospore germination was best at temperatures 10-15°C and Berner et al. (2015) and Demers et al. (2006) indicated optimal urediniospores germination occurred at 20°C. For germination of the spores they should be kept in the dark for at least 48 hours (French & Lightfield, 1990; Subrahmanyam et al., 1988; Turner et al., 1986), as it appears aeciospores, urediniospores and teliospores are light sensitive leading to reduced germination (Subrahmanyam et al., 1988; Turner et al., 1986). Germination of the different spore types of *P. punctiformis* increases in high relative humidity (Berner et al., 2015). To enhance penetration of a spore, a surfactant such as Tween 20 is often added to spore suspensions to aid in the breakdown of the leaf cuticle making it easier for pathogens to infect the plant. Tween 20 also helps to reduce the amount of wax on the leaves which will aid in navigation of stomatal hyphae to stomatal openings (Solymosi & Nagy, 1998; Thomson & Moeller, 1983).

The aim of this chapter is to determine the most effective method for infection of *C. arvense* with *P. punctiformis.* Being able to bulk up the pathogen would aid in further experiments. The determined infection method may give an indication of how the plant and pathogen interact.

5.2 Material and Methods

5.2.1 Infection Method

In July 2017 *C. arvense* root fragments approximately 5cm in length with two visible adventitious shoot buds, were planted in 750 ml pots containing a standard potting mix (Appendix C.1). Plants were then placed in a glasshouse at Lincoln University, with natural light conditions and temperatures 15-25˚C. One *C. arvense* genotype (69) collected as a single ramet from a pasture in Te Kopuru, Poutu Peninsula, Northland New Zealand, was used for this trial.

The trial used a plant extract that was prepared on the day of treatment. A plant extraction was made by collecting leaves from a genotype 69 plant which was ground using a mortar and pestle. The liquid was poured through microfilm to remove any plant material and the filtrate was used at 5% in the applied solution.

Spores used in the trial were collected in March 2017 from the Clyde site in Canterbury and stored in 30% glycerol in a -80˚C freezer. The spore suspensions applied were made to 6.8 x 10^5 spores per mL and consisted of urediniospores (95%) and teliospores (5%). The percentage of urediniospores and teliospores were determined by counting 100 spores in five replicates. Spore viability was checked by incubating 20µl of the suspension on an elevated microscope slide placed within a petri dish containing water for 24 hours at room temperature in natural daylight. Germination was counted

when the germ tube was at least the length of the spores' width. The spore suspension was applied to both the upper and lower surface of the leaves with a hand atomiser. The density of spores applied per 1mm² was determined (45 spores per 1mm²) by placing a blank slide with agar on the surface of the soil within the pot.

The experiment was conducted at the start of September and was undertaken when plants had been grown under glasshouse conditions for 6 weeks until they were 40mm high. During this time, plants were sprayed with Karate Zeon (0.2ml per 5ml) for aphids. Twenty-four hours prior to the application of the trial treatments, plants were watered.

In total there were 40 clonal replicate plants, with eight plants for each treatment. The five treatments were: Treatment 1- spore suspension; Treatment 2- spore suspension made with 0.1% Tween 20 (Labchem); Treatment 3- spores suspension made with 5% plant extract; Treatment 4- spores suspension made with 0.1% Tween 20 and 5% plant extract; Treatment 5- water only. For each Treatment, 3ml were applied to each individual plant. Following is the preparation of the specific components for the treatments.

After the treatments were applied, clear plastic bags were placed over each potted plant individually and placed in a dark growth room for 72 hours. After 72 hours the bags were removed and plant kept in the growth room for a further four days at 20°C and 16/8 light/dark. Plants were watered two to three times a week, or when required. After a total of one week in the growth room, plants were placed into a glasshouse in a randomized split-block design, where the temperature was approximately 15°C to 25°C, at the start of September and the light was 11/13 light/dark, end of September 13.5/10.5 light/dark this was the natural daylength hours.

Each week the plants were monitored and the following was recorded: the height of the plant (mm), the number of leaves per plant, number of shoots and the presence of *P. punctiformis* pustules. Four weeks after inoculation, one basal leaf was collected from four plants per treatment. The leaves were placed in FAA (formaldehyde 10% acetic acid (glacial) 10%, alcohol 50% and H_2O 35%) to de-stain for at least a week. The leaves were then inspected under a microscope to observe any infection. To observe internal infection, the leaves were cut into thin slithers and dyed with lactophenol cotton blue stain. Photos of signs of infection, spores or germination within the leaf material were taken.

Four weeks after the application of the treatment, plant material 30cm above the soil was cut with sterile scissors and reapplied with Treatment 1 to 5 listed above, where the spore suspensions were 9.8×10^4 spores/ml.

Eight weeks after inoculation, final recordings were taken and plants were harvested by cutting the plant at soil level. Leaves that appeared to have *P. punctiformis* infection were collected. Where no visible signs were observed, a leaf from the base of the plant and newer leaf at the top of the plant, was collected. Above soil plant material was cut and placed into paper bags. Roots were then washed and placed in separate paper bags from the above ground material. Shoots and roots were dried in the oven separately for 72 hrs at 60°C.

5.2.2 Molecular work

After the plants were dried, the above ground material and the roots were ground separately with a mortar and pestle in liquid nitrogen into a fine powder. Chelex DNA extraction was used to extract DNA. Three subsamples were taken from each plant, above ground material and roots. 30-40mg of dried plant material were transferred to 1.7mL tubes with 300 µl aliquot of a 10% (wv) Chelex 100 (BioRad) solution. Tubes were vortexed for 10 s three times and then placed into a heating block at 100°C for 10 min. The tubes were then vortexed for a further 10 s three times and returned to the heating block for 10 min at 100°C, then centrifuged for 10 min at 13,000 × g. The supernatant (150µl) was removed and placed into 0.6mL ml tubes. DNA concentration was measured using a Nanodrop spectrophotometer. Samples were then diluted as needed to a concentration of 15-25 ng of DNA.

Molecular means were used to determine if *Puccinia* was present. *Puccinia* specific primers were used, PuncF 5' ACCCCTAACACTTGTTTGTG 3' and PuncR 5' GCACTAAAGGTATTGGCAAG 3' (Berner et al., 2015). Reactions were undertaken in 20 µl, each tube contained 15-25ng of DNA template, 10 µl of DreamTaq Green PCR Master Mix (ThermoFisher), 1 µM of each Punc primer (forward and reverse) and the remaining volume, H_2O. The cycle parameters were, an initial denaturation of 94 °C for 2 min, 30 cycles, denaturing for 30 s at 94 °C, annealing at 62°C for 3 min and extension at 72 °C for 2 min. Final extension, 10 min at 72°C (Berner et al., 2015). PCR products were separated in a 1% agarose gel with 1kb+ ladder and visualised after staining with ethidium bromide (0.5µg/mL) for 45 min, rinsed in water for 15 min and then exposed to UV light. A positive control was used using spore only *P. punctiformis* DNA extract (10-20ng). Bands presence confirmed the presence of *P. punctiformis.* Plants that appeared to have symptoms of *P. punctiformis* at harvest were run first with different dilutions of DNA extract. In total there were three replications, each replication had 80 samples, 40 leaf samples and 40 roots samples, in total 240 samples.

5.3 Results

5.3.1 Glasshouse infection

The percentage of urediniospores and teliospores was 95% and 5% respectively. The percentage of spore germination was recorded 10-25%, on average of 15% germination. *P. punctiformis* symptoms were not seen in the first six weeks. When half the plants were cut at 4 weeks, the leaves were examined under a compound microscope. No sign of *P. punctiformis* was observed in these leaves. During the last two weeks possible signs of *P. punctiformis* symptoms appeared, out of the 40 plants only three appeared to have *P. punctiformis* pustules*;* treatment 1.2 replications 1-3, spore suspension and cut at 4 weeks then reapplied with spore suspension. Treatment 1.1 the same treatment as 1.2 but not cut showed no signs of infection. Plant 1.2 replication 1, there appeared to be dark orange/brown pustules (Figure 5.1). These spore pustules were located on the underside of the leaf across both sides of the main vein of the leaf going from the centre to the outer edges (Figure 5.1). The older leaves of the plants had died throughout the 8 weeks of growth. Glasshouse temperatures were recorded and the range varied from what was expected (15-25°C), the temperatures recorded ranged from 14.9-33°C.

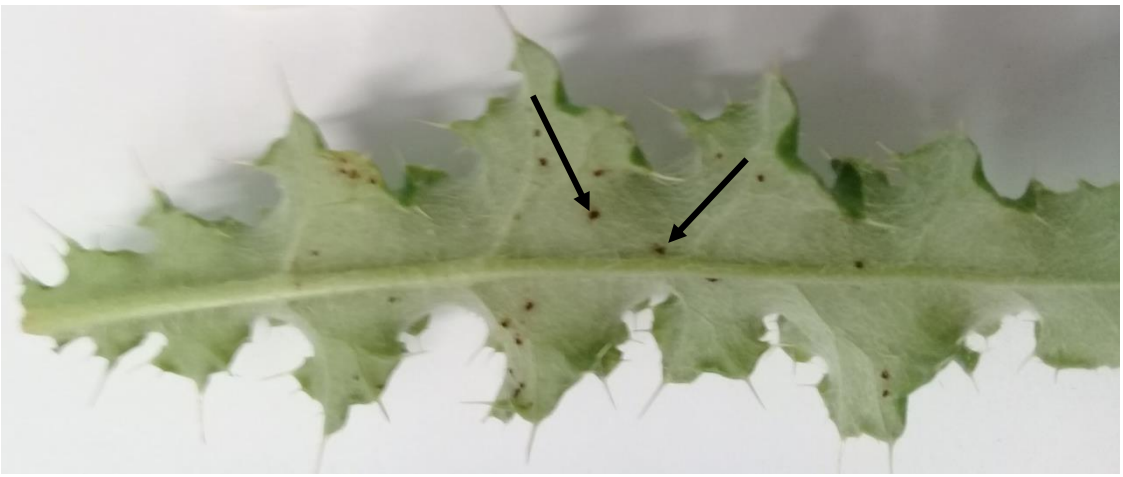

Figure 5.1: Glasshouse infection plant leaf 1.2 replication 1. Possible visual symptoms of *Puccinia punctiformis* on the underside of a *Cirsium arvense* leaf. Dark orange/brown pustules spread across the underside of the leaf indicative of localised infection. Arrows point to two *Puccinia punctiformis* pustules.

5.3.2 Microscopic work

No *P. punctiformis* spores were present on or in the leaves collected at 4 weeks by microscopic techniques. Leaves collected at harvest were found to have spores present. Spores were present inside three of the 80 plants. There were all in Treatment 1.2, spore suspension and cut at 4 weeks replications 1-3 (Figure 5.2). Plants of the same treatment, but were not cut at 4 weeks, showed no

spores inside the leaf. Both teliospores and urediniospores spores were observed (Figure 5.2). More urediniospores are seen inside the leaves compared to teliospores. Figure 5.2 a) and b) indicates a uredinium containing urediniospores whilst c) and d) indicate a urediniospore and a teliospore.

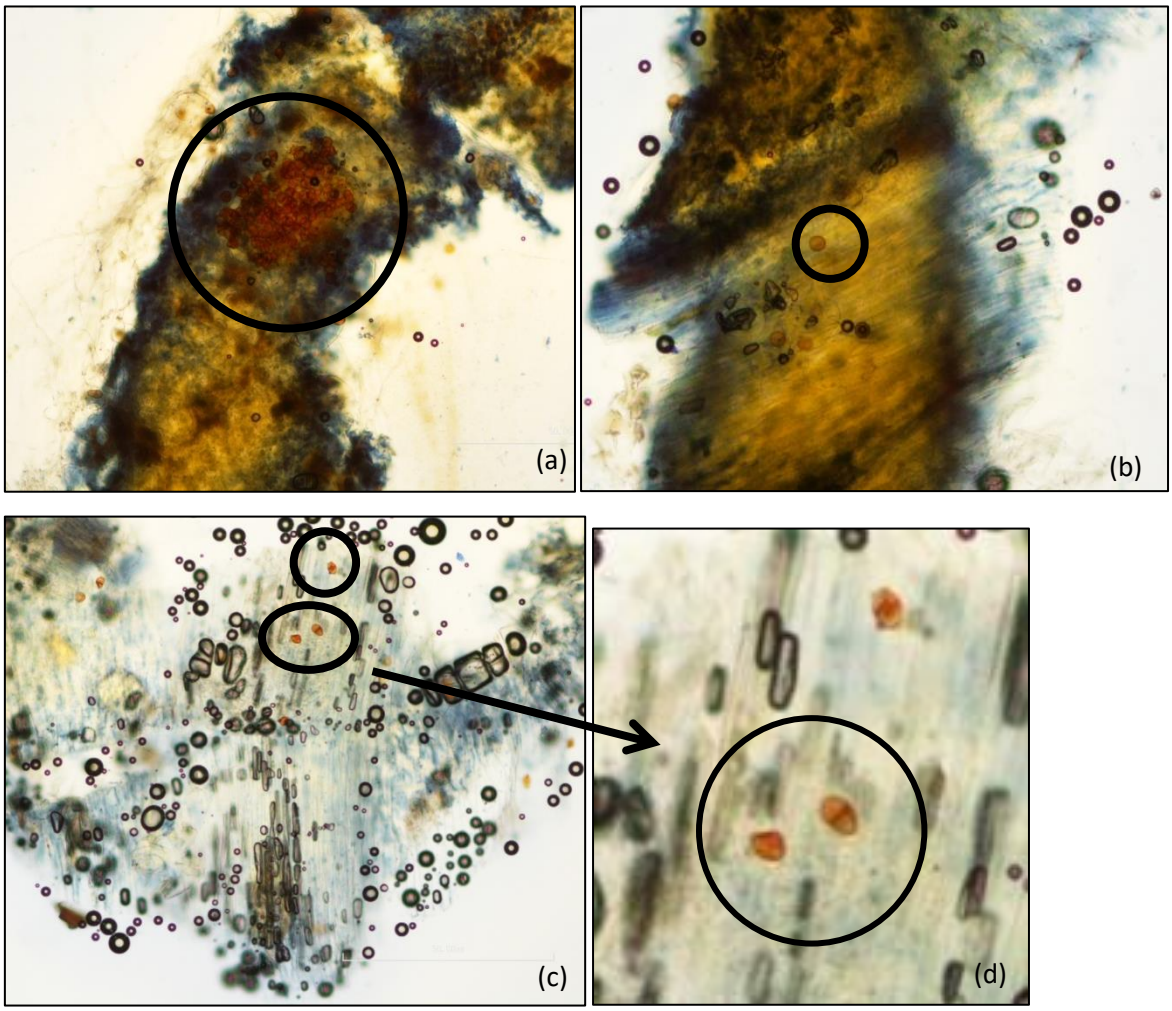

Figure 5.2: Microscopy on leaves from glasshouse infection. (a) is treatment 1.2 rep 1, Spore structure and both urediniospores and teliospores and (b & c) 1.2 rep 2 both urediniospores and teliospores. (d) Close up of picture c looking at the two types of spores, urediniospores (left) and teliospores (right). Circles indicate spores.

5.3.3 Molecular work

The Chelex DNA extraction method yielded high DNA samples that needed diluted and ranged from 1-1.6 A260/280. PCR with *Puccinia* specific primers resulted in no amplification of products with no bands visible on the gel from the leaves or roots of the glasshouse plants (Figure 5.3). Dilutions of samples that had *P. punctiformis* pustules showed no bands. The positive control did amplify a band at 460 bp showing that the protocol was successful.

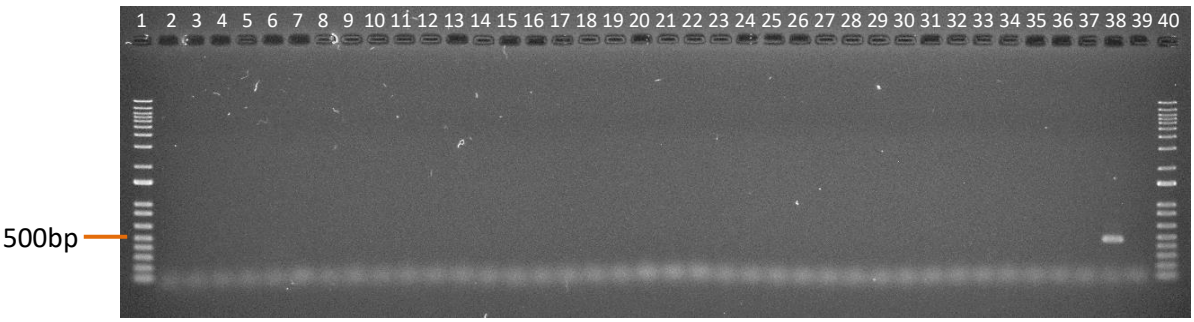

Figure 5.3: 1% agarose gel of a portion of samples of glasshouse infection plants. First (1) and last lane (40) 1 kb+ ladder. Lane 38 positive control with rust spores, lane 39 negative control. Lanes 2-36, 1.1 R1-R4, 1.2 R1-R4, 1.1 R1R-R4R, 1.2 R1R-R4R, 2.1 R1-R4, 2.2 R1-R4, 2.1 R1R-R4R, 2.2 R1R-R4R and 3.1 R1-R4. R1-R4 are leaves replications 1-4, R1R-R4R are roots replications 1-4.

5.4 Discussion

Puccinia punctiformis can infect *C. arvense* systemically and localised with systemic infection having the potential to cause the most damage (Berner et al., 2013; French & Lightfield, 1990). There have been many studies on the infection of *P. punctiformis* and the different factors that influence the amount of infection on *C. arvense*. The optimal temperature for teliospores is 15˚C (Anikster, 1986; Thomas et al., 1994; Watson & Keogh, 1980) and the optimal temperature for urediniospores is 20 ˚C (Berner et al., 2015; Demers et al., 2006). For urediniospores and teliospores, germination increased in darkness (French & Lightfield, 1990; Subrahmanyam et al., 1988; Turner et al., 1986) and in high relative humidity (Morin et al., 1992). Mowing has also been seen to increase *P. punctiformis* incidence in *C. arvense* sites compared to sites where thistles were not mown (Demers et al., 2006). This study showed that infection only occurred in one treatment, treatment 1.2, which was a spore suspension only with no added surfactant (Tween20) or plant extract, then subsequently the plant was cut at four weeks and additional spores were applied. This could suggest that plant extract and surfactants may inhibit *P. punctiformis* infection and growth (Czarnota & Thomas, 2013; Schulze & Schönherr, 2003). Spores were observed microscopically, however, the molecular work indicated no rust was present which suggest there may be inhibition of the PCR as there were spores present in the samples extracted for DNA.

5.4.1 Microscopic and molecular work

The glasshouse infection method resulted in very little infection. Microscopy showed that for treatment 1.2, spores were present within the leaves (Replication 1-3). Visual symptoms of *P. punctiformis* pustules were also observed. Molecular work showed that there was no rust infection despite what the microscopic work showed. Despite implementing different treatments to induce plant infection, there was very little overall infection observed. As a result of poor infection from this

experiment, the best method for infection cannot be ascertained. Only one treatment had infection (treatment 1.2) thus no comparisons can be made regarding the most appropriate infection method of *C. arvense* plants with *P. punctiformis.*

Inoculation of the pathogen in a glasshouse environment had been conducted, as the pathogen cannot be cultured in the lab as easily as necrotrophic fungi. During this trial, plastic bags covered the individual thistles that were placed into a dark growth room during the first 72 hours, because three types of spores (urediniospores, aeciospores and teliospores) are light sensitive and therefore darkness would be expected to increase germination (Berner et al., 2015; French & Lightfield, 1990; Subrahmanyam et al., 1988; Turner et al., 1986). During the first week, the thistles were kept in a growth room at 20˚C after application of the pathogen as this was the optimal temperature for urediniospore germination and infection (Berner et al., 2015; Demers et al., 2006). After the first 4 weeks post the initial application of spores, thistle leaves from half of each treatment were cut to 30cm above soil level; this follows the study indicating that mowing increases *P. punctiformis* infection (Bourdôt et al., 2011; Demers et al., 2006). The cut thistles were then reapplied with the same treatment. The optimal conditions we produced from gathering information from various literature and studies. Different treatments were used to enhance the incidence of *P. punctiformis* infection on *C. arvense* plants in the glasshouse. Despite the information sought from published work successful infection was still unable to be obtained.

The microscopic techniques visually assessed the leaves that were collected at harvest. Only three plants had symptoms at the end of the 8 weeks, all from treatment 1.2, spore suspension and cut at 4 weeks. It was thought that treatment 1 would be the treatment less likely to have *P. punctiformis* infection as the other treatments had either Tween 20, which breaks down the leaf cuticle and aids in spore infection or plant extract, which is thought to increase germination. Cutting the thistles at 4 weeks was hypothesised to increase infection, as this adds another entry point and perhaps spreads spores that are already present and simulates mowing which is thought to increase incidence of *P. punctiformis* infection (Bourdôt et al., 2011; Demers et al., 2006). Therefore treatment 1.2 is more likely to show infection compared to treatment 1.1 where the thistles were not cut during the 8 weeks of growth. Spores were within the plant and some germination had occurred as well as the indication of a spore structure, a uredinium containing urediniospores. No spores were seen on or in the leaves at four weeks. The first initial application of the spores could have been washed off and therefore the reapplication of the spore suspension may have increased chance of infection due to the increase in spores. Temperatures in the glasshouse varied from 16-23.5˚C which should not have damaged the spores however there were some days where the temperature reached 30˚C which could have damaged and killed the pathogen; perhaps explaining why no spores were seen after 4 weeks. Temperatures in the last 4 weeks had days where it also got to 30˚C which could also explain

why a minimal number of plants (three) were seen to have spores (Berner et al., 2015; Thomas et al., 1994).

The molecular work with *Puccinia* specific primers were unable to detect *P. punctiformis* in the plant material. There could be several reasons as to why this may have occurred. Firstly, *P. punctiformis* infection is minimal in the thistles and therefore when taking a subsample from the plant, *P. punctiformis* spores may not be present in the sample, three subsamples minimise this error. The second reason the molecular work showed no spores present is that the levels of spore DNA in the extraction may have been minimal. However, from previous experiments and molecular work (Chapter 3), the primers are known to pick DNA levels as low as 0.09ng which equates to 27 nuclei or 25 urediniospores (95%) and 1 teliospores (5%). Therefore, perhaps levels may have been below the detection threshold and hence a more sensitive molecular technique is needed. The experiment in Chapter 3 was undertaken with rust spores only, so it is unknown whether plant material interferes with the primers and inhibits the reaction. In Berner et al. (2015) fresh leaves were used and there appeared to be no inhibition of the primers, the plant material that was used in this trial was dried and ground with liquid nitrogen or had been de-stained in FAA which may have produced different results compared to Berner et al. (2015). It is also difficult to determine how much *P. punctiformis* DNA is present in the extracted DNA because the nanodrop reads all DNA present which may have only been plant DNA. That is why the PCR protocol was also run on DNA extraction from leaves that appeared to have *P. punctiformis* symptoms with different dilution factors and again no bands were present.

Treatments 3 and 4 with plant extract had no *P. punctiformis* present microscopically or molecularly, this could be because the plant extract was processed by damaging leaves which may produce volatiles that is not conducive for biotrophic fungi. *P. punctiformis* is a biotrophic fungus and may not germinate and cause subsequent infection in the presence of such plant material. The process used in this study may have reduced the amount of infection and therefore future work should use plant volatiles (Connick & French, 1991; French et al., 1994). Plant volatiles can increase the germination of teliospores and this increased the infection of *P. punctiformis* in *C. arvense* (Connick & French, 1991; French et al., 1994). In several studies mowing is thought to increase spread and infection, however, it is unclear as to why mowing increases spread and infection (Bourdôt et al., 2011; Demers et al., 2006). One reason of increased infection could be because when mowing occurs in a patch, spreading spores caused by the disturbance of the plants or mowing adds an alternative entry point or a combination (Bourdôt et al., 2011; Demers et al., 2006). This is indicated by treatment 1.2 which had *P. punctiformis* symptoms. Treatment 1.2, plants were cut at 4 weeks and then reapplied with the spores, simulating the mowing method. This could indicate that the mowing method at a larger scale increases spread and infection. Treatments 2 and 4 had spore suspensions with Tween 20,

which is a surfactant that breaks down the leaf cuticle and reduces the thickness of the wax layer (Czarnota & Thomas, 2013). This could increase infection as it is easier for the spore to germinate and navigate towards the stomata and infect (Solymosi & Nagy, 1998; Thomson & Moeller, 1983). *P. punctiformis* can detect the height of the ridges of the leaves and stomata for appressorial development and infection (Allen et al., 1991; Hoch & Staples, 1987). However, in this case, the surfactants could have inhibited the infection of *P. punctiform.* Surfactants can cause damage to plants if the wrong type or concentration is used (Czarnota & Thomas, 2013). Other papers used surfactants such as Tween 20 or Polysorbate 20 and had low levels of *P. punctiformis* infection on *C. arvense*, which suggests that this may not be a limiting factor (Berner et al., 2015; Müller et al., 2011).The types of spores that were applied to the thistles and the percentage of these spores may have affected the amount of infection or the type of infection that occurred. More localised infection may have been seen which is a result of urediniospores spreading and infecting. There were only 5% of teliospores which may have led to less systemic infection. The fungus overwinters in the roots as a result from hyphal growth from teliospores (Berner et al., 2013; French & Lightfield, 1990). If there was perhaps more teliospores in the spore percentage perhaps there may have been a more intensive infection and less localised infection.

5.4.2 Limitations and further work

There are several limitations of this experiment that occurred, including the time of year the experiment was conducted which was at the end of winter. The measured glasshouse temperatures ranged from 14.9°C to 33°C. The varying temperatures may have affected the infection of *P. punctiformis.* At the higher temperatures, the pathogen is more likely to die (Demers et al., 2006; Thomas et al., 1994). Germination of the spores in the first week should not have been affected by the temperatures as they were in a growth room set at 20°C. For optimal conditions plants would be best to be in the growth room throughout the whole experiment, but this was not done in this study due to the lack of space in the growth rooms. Growth room temperature and lighting conditions are consistent and controllable reducing the amount of variability that was seen in the glasshouse. Another limitation of this experiment is the age of spores used in the suspensions. The collection of *P. punctiformis* spores occurred at the end of the previous season (March) and kept in 30% glycerol in a -80°C freezer for five months. Higher amounts of germination and infection may have occurred if spores were fresh. Watson and Keogh (1980) used fresh urediniospores and had high germination rates, however Wandeler et al. (2008) stored urediniospores at -20˚C in airtight plastic vials for 2 weeks and still had 30% germination. French and Lightfield (1990) stored teliospores at 4˚C for up to three years and had germination. Perhaps storing spores at colder temperatures (-20˚C) reduced germination compared to warmer temperatures (-4˚C). There was little infection and germination in the majority of the plants, the plants may have needed a longer period of time for germination and

infection. Due to the low germination percentage, the *P. punctiformis* spores may have needed longer to infect and germinate the thistles. Plants could be left for a longer period to allow for more time for *P. punctiformis* to successfully infect, French and Lightfield (1990) observed greater plant infection occurred three months after application. There is also limitations with the molecular work as it is unknown whether the plant phenolics may have inhibited the primers resulting in no amplification of the *Puccinia* product (De Boer et al., 1995). All the samples had plant material present which may explain why even the samples with *P. punctiformis* present had no bands present in the molecular work.

Future studies could include treatments such as inoculating the roots directly. Directly infecting the roots is another method that is thought to increase the systemic infection (Frantzen & Van der Zweerde, 1994; French et al., 1994) as it is known that the mycelium can overwinter in the roots and then when the thistle shoots emerge they are already systemically infected (Berner et al., 2013; French & Lightfield, 1990). Looking at the different types of surfactants could be another treatment to see if there is any difference in infection, which will help with later experiments or with augmentative application (Czarnota & Thomas, 2013; Schulze & Schönherr, 2003).

5.5 Conclusion

This experiment aimed to determine the best method to artificially infect the *C. arvense*, which could aid in future experiments. Successful artificial infection allows the biotrophic pathogen to be bulked up on a living host. There was very little infection and thus no infection process can be recommended. Only 3 out of 32 treated thistles had *P. punctiformis* symptoms of infection. Microscopic techniques showed that 3 out of the 4 plants from treatment 1.2, spore suspension and cut at 4 weeks, had infection. This does indicate that cutting the thistles may increase infection of the pathogen. It was thought that the surfactants and plant extract would aid in infection, but this was not observed. Treatments that had either Tween 20 or plant extract may have prevented infection instead of increasing infection. The molecular work showed that there was no *P. punctiformis,* possibly because there were low levels of the pathogen in the subsampling or a result of plant material inhibiting the molecular PCR protocol. A successful method of infection is required to aid in further studies of the *P. punctiformis* and *C. arvense*.

Chapter 6

Concluding discussion

This research has conducted several experiments in the aim to determine if the observed differences in infection of *Cirsium arvense* were attributable to genetic variation between *Puccinia punctiformis* isolates in New Zealand. In addition, it has also looked at the concentration of *P. punctiformis in planta,* as well as setting out to determine the most effective infection method for the rust.

C. arvense is a weed found throughout New Zealand and is problematic, especially in agricultural systems (Cripps et al., 2009). *P. punctiformis* is a rust fungus that only infects *C. arvense*. In the field, the biotrophic fungus systemically infects the thistle and subsequently reduces shoot abundance (Berner et al., 2013; Cripps, 2016). *P. punctiformis* has the potential to be a successful biological control agent, however, there is little known about its genetic diversity, whereas *C. arvense* is known to be highly genetically diverse (Bommarco et al., 2010; Hettwer & Gerowitt, 2004).

In this study, a survey was conducted in the North and South Island of New Zealand at 22 different *C. arvense* sites, with the aim of determining the varying levels of *P. punctiformis* infected shoots in New Zealand. For each *C. arvense* population, nine $1m^2$ quadrats were surveyed. Within each quadrat, the total number of thistles and the number of *P. punctiformis* infected shoots were recorded. Shoots were sampled for later processing. *P. punctiformis* was present in all 22 sites surveyed throughout New Zealand. The Ruakura site, in the Waikato in the North Island, had the highest amount of *P. punctiformis* infected shoots (11.1%) within the surveyed area. Six other sites (Manutuke, Happy Valley, Micklesons, Wai-iti Rd, Bushfield and Clyde) had high levels of infected shoots that did not differ significantly ($P>0.05$) to the Ruakura site. Eight out of 22 sites had no *P. punctiformis* infected shoots within the nine surveyed quadrats. The percentage of *P. punctiformis* infected shoots varied within and between locations, with no apparent trend between the populations. The different *C. arvense* sites have varying climatic and habitat ranges. There were no obvious characteristics between the populations exhibiting higher levels of *P. punctiformis* infection compared to those with low or nil infected shoots. Not all regions and areas of New Zealand were surveyed, such as the west coast of the North and South Island which also present different climatic habitats. Surveying more sites may indicate further *P. punctiformis* infection in *C. arvense* across New Zealand. Characterisation of the genetics of the plant and pathogen may give an indication why the levels of *P. punctiformis* infection varies between populations throughout New Zealand (Hodgson, 1973; Moore, 1975; Ozcan et al., 2015; Turner et al., 1981).

The infected shoots of *P. punctiformis,* collected from the 22 surveyed sites, were analysed to determine if any genetic differences between isolates were apparent. The PCR (Polymerase Chain Reaction) used *Puccinia* specific primers to amplify the ITS (Internal Transcribed Spacer) region (Berner et al., 2013; Gil-Lamaignere et al., 2003; Miranda et al., 2010). This confirmed that *P. punctiformis* was present in the samples. PCR products of the ITS region were either sequenced or processed using RFLP (Restricted Fragment Length Polymorphism) (Rasmussen, 2012a). Spores were collected from 5 of the 22 surveyed populations and analysed for genetic variation using RAPD-PCR (Random Amplified Polymorphic DNA-PCR) (Feng et al., 2009; Noonan et al., 1996). Amplification of the ITS region showed that not all extracted samples had *Puccinia* present which suggested the plant material may have interfered with the PCR reaction. Some *P. punctiformis* samples were extracted from old plant material. The degradation of plant material and phenolics present may have inhibited the primers resulting in no amplification of the *Puccinia* product (De Boer et al., 1995). Collection of spores while the plant is fresh, or the use of more sensitive and specific primers, could reduce the amount of interference that occurred. RFLP found no genetic difference between *P. punctiformis* isolates. Not all fragmented bands were visible on the gel, therefore a higher quality of agarose gel may allow for identification of the smaller bands, as it is difficult to detect fragments smaller than 20bp (Poly et al., 2001). Sequencing of the ITS region found one (Hurst) out of 11 isolates had genetic differences (in total 6 bp substitutions). The RAPDs, which amplifies polymorphisms over the entire genome, indicated genetic differences within and between populations. The genetic difference found in this study could indicate that perhaps there is genetic mutation or sexual reproduction occurring within *P. punctiformis* populations. A more comprehensive number of samples are required to indicate whether further genetic diversity between *P. punctiformis* isolates is evident in New Zealand. The genetic diversity shown in this study could partly explain why there is variation in infection levels between *C. arvense* populations (Agrios, 2005).

In this study, the qPCR (quantitative polymerase chain reaction) molecular technique determined the concentration of *P. punctiformis* in *C. arvense* (Arya et al., 2005; Saikia & Kadoo, 2010). Nine plants were collected from the Clyde site in Canterbury in the South Island. Six plants with visible *P. punctiformis* pustules and three plants with no visible symptoms were collected (Thomas et al., 1994). The amount of *P. punctiformis* was determined in specific plant parts, from the top to the bottom of the shoot. The general trend found that the highest concentration of *P. punctiformis* was located at the top of the plants and decreased towards the base. There were significant differences in the *P. punctiformis* concentration between plants as well as within a plant, with apparently younger tissue having the greater quantity of rust. Plants that had no visible symptoms had lower amounts of *P. punctiformis* compared to plants that had symptoms. Low levels of *P. punctiformis* were obtained in asymptomatic plants suggesting infection may be in the early stages or the plant

has induced a defence response to the pathogen. These results were from plants that were collected at a point in time and therefore the stage of infection is unknown. The life cycle of *C. arvense* may also influence the amount of *P. punctiformis* present (Moore, 1975). The morphological characteristics of *C. arvense* could be a factor, influencing the amount of *P. punctiformis* in a particular location within different sections of the plant. These characteristics include the thickness of the wax cuticle, stomata density and hairiness of leaves (Buschhaus & Jetter, 2012; Hodgson, 1973; Moore, 1975; Ozcan et al., 2015; Solymosi & Nagy, 1998). *C. arvense* is genetically diverse and is known to have a wide range of clonal differentiation (Bommarco et al., 2010; Hettwer & Gerowitt, 2004). The various levels of *P. punctiformis* in the different plants could be attributed to this genetic diversity of *C. arvense.* A more intense sampling could occur at different stages of both the plant and pathogen life cycle. Sampling both the roots and plant may indicate if the pathogen has overwintered in the roots or whether it is only present in the shoots. Understanding where the *P. punctiformis* is located within *C. arvense* may aid in understanding the host-pathogen interaction and how the pathogen infects the plant as well as how it moves within the plant (Green & Bailey, 2000; Ozcan et al., 2015).

During this study, a glasshouse trial was conducted to determine the most effective infection method of *C. arvense* with *P. punctiformis.* The trial was conducted in a glasshouse at Lincoln University, Canterbury. One genotype of *C. arvense* was grown and then applied with five treatments; treatments 1-4 were various mixtures of spore suspension, Tween 20, plant extract and cut after 4 weeks, and treatment 5 was water. Both microscopic and molecular techniques were carried out to determine if any infection occurred. Very little infection was found and therefore the most effective infection method was unobtainable. The microscopy investigation indicated three out of the 32 treated plants became infected by the *P. punctiformis* spores. The three plants were all from treatment 1.2 where plants applied with a spore suspension and subsequently cut at 4 weeks, with further spore suspension applied. However, the molecular techniques showed that there was no *P. punctiformis* present in any of the plants for all of the treatments in either the root or shoot/leaves. This suggests that plant material may interfere with the PCR protocol (De Boer et al., 1995; Schrader et al., 2012). The spores used in this experiment were collected at least 6 months prior to application of treatments and stored in glycerol, this may have reduced the number of viable spores. Fresh spores may increase the amount of germination and therefore increase the amount of infection. Obtaining a successful infection method will allow for easier bulking of the pathogen which will help with later experiments or with augmentative application.

This research has determined the presence of *P. punctiformis* in all *C. arvense* populations surveyed in New Zealand, even at very low levels. This study found genetic differences between *P. punctiformis* isolates located around New Zealand both within the ITS region and the whole genome.

Further studies can look at the genetics of both *P. punctiformis* and *C. arvense* which may aid in understanding the interaction between the host and pathogen. This may indicate the reason for differences in infection between *C. arvense* populations. Further studies should investigate the concentration of *P. punctiformis* in *C. arvense* at different stages of the life cycle of both the pathogen and the plant. Alongside this experiment, the determination of the spore types present, teliospores or urediniospores, within a single plant should be undertaken. The infection of the plant by the different spore types needs to be ascertained prior to future glasshouse experimentation. Determining the most effective method of infection will aid in later experiments that may help in understanding *P. punctiformis* and its interaction with *C. arvense.*

This study has contributed to the knowledge of the possibility of *P. punctiformis* as a biological control agent for *C. arvense*. The research has added recent data of the distribution and density of *P. punctiformis* infection in different *C. arvense* populations across New Zealand. There were only 22 sites that were surveyed and only in certain areas which leaves some areas of New Zealand with no data on the populations density of both the pathogen and weed. Surveying more sites in varying locations would have been more beneficial but time and money was limiting. The genetic differences of the *P. punctiformis* was studied as there is a lack of knowledge in this area, the molecular work indicated that there is a genetic difference between *P. punctiformis* samples collected around New Zealand. The indication of genetic differences of *P. punctiformis* contributes to the understanding that this may be a factor in the varying levels of infection in *C. arvense* populations. There were a limited number of samples that were genetically tested, thus more samples would increase the information regarding the genetic differences and genome of *P. punctiformis*. In this thesis a successful infection method was not found for *P. punctiformis* this makes it complicated to do further studies. The study of *P. punctiformis in planta* has increased the knowledge of how the pathogen may move within the plant and where the higher concentrations are located. The overall contribution of the studies has gained more understanding on the interaction between *C. arvense* and *P. punctiformis.* Further knowledge that needs to obtained for *P. punctiformis* to be considered to be in a successful biological control programme as it is still unknown why there is such a varying level of *P. punctiformis* infection in different *C. arvense* populations across New Zealand.

Appendix A

A.1 List of samples collected from around New Zealand, number given to the sample, the region samples collected, Survey and sample collection area, the spore removal method.

Isolate #	Region	Area	Method
1	Gisborne	Manutuke T1 Q1 S1	Spores
2	Gisborne	Manutuke T3 Q1 S4	Spores
4	Gisborne	Manutuke T1Q2 S4	Spores
5	Gisborne	Manutuke T2 Q1	Spores
6	Gisborne	Manutuke T1 Q2 S3	Spores
7	Gisborne	Manutuke T2 Q2 S3	Spores
8	Gisborne	Manutuke T3 Q1 S5	Spores
9	Gisborne	Manutuke T3 Q1 S1	Spores
10	Gisborne	Manutuke T1 Q2 S2	Spores
11	Gisborne	Manutuke T1 Q2 S1	Spores
12	Gisborne	Manutuke T3 Q1 S6	Spores
13	Gisborne	Manutuke T3 Q1 S2	Spores
14	Gisborne	Manutuke T3 Q2	Spores
15	Gisborne	Manutuke T2 Q3	Spores
16	Gisborne	Manutuke T1 Q3	Spores
17	Gisborne	Manutuke T2 Q2 S1	Spores
18	Gisborne	Manutuke T2 Q2 S1	Spores
19	Gisborne	Manutuke T3 Q3	Spores
20	Bay of Plenty	Highlands 1 T2 Q3	Leaf
21	Bay of Plenty	Highlands 1 T1 Q3	Leaf
22	Bay of Plenty	Highlands 1 T3 Q3	Leaf
23	Manawatu-Wanganui	Micklesons T2 Q3 S1	Leaf
24	Manawatu-Wanganui	Micklesons T3 Q3 S1	Leaf
25	Manawatu-Wanganui	Micklesons T3 Q1 S1	Leaf
26	Manawatu-Wanganui	Micklesons T2 Q1 S1	Leaf
27	Manawatu-Wanganui	Micklesons T3 Q2 S2	Leaf
28	Manawatu-Wanganui	Micklesons T1 Q1 S2	Leaf
29	Manawatu-Wanganui	Micklesons T2 Q2	Leaf
30	Manawatu-Wanganui	Micklesons T3 Q2 S1	Leaf
31	Gisborne	Manutuke T3 Q1 S3	Spores
32	Manawatu-Wanganui	Micklesons T1 Q3	Leaf
33	Manawatu-Wanganui	Micklesons T1 Q1 S1	Leaf
34	Manawatu-Wanganui	Micklesons T1 Q2 S1	Leaf
35	Manawatu-Wanganui	Micklesons T1 Q2 S3	Leaf
36	Canterbury	Clyde T3 Q1	Spores
37	Canterbury	Clyde T2 Q2	Spores
38	Canterbury	Clyde T3 Q2	Spores

39	Canterbury	Clyde T1 Q3	Spores
40	Canterbury	Clyde Extra 2	Spores
41	Canterbury	Clyde Extra 1	Spores
42	Canterbury	MacDonald T2 Q2	Spores
43	Canterbury	MacDonald T1 Q3	Spores
44	Canterbury	MacDonald T2 Q3	Spores
45	Canterbury	MacDonald T1 Q1	Spores
46	Canterbury	Hurst T2 Q3	Spores
47	Canterbury	Hurst T1 Q3	Spores
48	Canterbury	Hurst Extra 2	Spores
49	Canterbury	Hurst T2 Q1	Spores
50	Canterbury	Hurst Extra 1	Spores
51	Otago	Smith T2 Q2	Spores
52	Otago	Smith T1 Q1	Water and tween
53	Otago	Smith Extra 1	Spores
54	Bay of Plenty	Highlands 1 T3 Q2	leaf
55	Waikato	Bridge 64 T2 Q2	Water and tween
56	Waikato	Bridge 64 T3 Q1	Water and tween
57	Waikato	Bridge 64 T1 Q3	Water and tween
58	Waikato	Bridge 64 Extra 1	Water and tween
59	Waikato	Bridge 64 T3 Q2	Water and tween
60	Waikato	Bridge 64 T2 Q3	Water and tween
61	Southland	Dunrobin Valley Extra 1	Water and tween
62	Southland	Dunrobin Valley Extra 2	Water and tween
63	Southland	Dunrobin Valley Extra 3	Water and tween
64	Southland	Dunrobin Valley T3 Q2	Water and tween
65	Southland	Dunrobin Valley T3 Q3	Water and tween
66	Southland	Happy Valley T2 Q2 S3	Water and tween
67	Southland	Happy Valley T1 Q2 S1	Water and tween
68	Southland	Happy Valley T1 Q1 S1	Water and tween
69	Southland	Happy Valley T2 Q2 S5	Water and tween
70	Southland	Happy Valley Extra 2	Water and tween
71	Southland	Happy Valley Extra 3	Water and tween
72	Southland	Happy Valley Extra 1	Water and tween
73	Southland	Happy Valley T2 Q2 S4	Water and tween
74	Southland	Happy Valley T1 Q3 S1	Water and tween
75	Southland	Happy Valley T2 Q2 S6	Water and tween
76	Southland	Happy Valley T2 Q2 S1	Water and tween
77	Southland	Happy Valley T1 Q1 S2	Water and tween
78	Southland	Happy Valley T2 Q2 S2	Water and tween
79	Southland	Happy Valley T2 Q1 S1	Water and tween
80	Southland	Milligans T1 Q1	Water and tween
81	Southland	Milligans T1 Q3	Water and tween
82	Southland	Milligans T3 Q3	Water and tween
83	Southland	Milligans T3 Q2	Water and tween
84	Southland	Milligans T2 Q3	Water and tween

85	Southland	Milligans T2 Q1	Water and tween
86	Southland	Milligans T2 Q2	Water and tween
87	Bay of Plenty	Highlands 2 T2 Q1 S1	Water and tween
88	Bay of Plenty	Highlands 2 T1 Q1	Water and tween
89	Bay of Plenty	Highlands 2 T2 Q3	Water and tween
90	Bay of Plenty	Highlands 2 T1 Q3	Water and tween
91	Bay of Plenty	Highlands 2 T3 Q3	Water and tween
92	Bay of Plenty	Highlands 2 TT3 Q2 S1	Water and tween
93	Bay of Plenty	Highlands 2 T1 Q2	Water and tween
94	Bay of Plenty	Highlands 2 T3 Q1	Water and tween
95	Bay of Plenty	Highlands 2 T2 Q2	Water and tween
96	Waikato	Poutu T3 Q2	Water and tween
97	Waikato	Poutu T3 Q3	Water and tween
98	Waikato	Poutu T2 Q3	Water and tween
99	Waikato	Poutu T1 Q3	Water and tween
100	Waikato	Poutu T1 Q1 S1	Water and tween
101	Waikato	Poutu T2 Q2	Water and tween
102	Waikato	Poutu T2 Q1 S1	Water and tween
104	Waikato	Poutu T1 Q1 S2	Water and tween
109	Manawatu-Wanganui	Sheridon T1 Q2	Water and tween
110	Manawatu-Wanganui	Sheridon T3 Q2	Water and tween
111	Manawatu-Wanganui	Sheridon T1 Q3	Water and tween
112	Manawatu-Wanganui	Sheridon T3 Q3	Water and tween
113	Manawatu-Wanganui	Sheridon T3 Q1	Water and tween
114	Manawatu-Wanganui	Sheridon T1 Q1	Water and tween
115	Manawatu-Wanganui	Sheridon T2 Q3	Water and tween
116	Manawatu-Wanganui	Sheridon T2 Q1	Water and tween
117	Manawatu-Wanganui	Duncans T2 Q3	Water and tween
118	Manawatu-Wanganui	Duncans T3 Q2 S1	Water and tween
119	Manawatu-Wanganui	Duncans T3 Q3	Water and tween
120	Manawatu-Wanganui	Duncans T3 Q1	Water and tween
121	Manawatu-Wanganui	Duncans T1 Q3 S1	Water and tween
122	Manawatu-Wanganui	Duncans T1 Q2	Water and tween
123	Manawatu-Wanganui	Duncans T1 Q1	Water and tween
124	Manawatu-Wanganui	Duncans T2 Q2	Water and tween
125	Manawatu-Wanganui	Duncans T2 Q1	Water and tween
126	Wellington	Gilbert Rd Extra 2	Water and tween
127	Wellington	Gilbert Rd Extra 1	Water and tween
128	Wellington	Gilbert Rd T1 Q1	Water and tween
129	Gisborne	Wi Pere T3 Q2	Water and tween
130	Gisborne	Wi Pere T3 Q3	Water and tween
131	Gisborne	Wi Pere T2 Q1 S1	Water and tween
132	Gisborne	Wi Pere T1 Q3 S2	Water and tween
133	Gisborne	Wi Pere T1 Q3 S1	Water and tween
134	Gisborne	Wi Pere T1 Q1	Water and tween
135	Gisborne	Wi Pere T1 Q2	Water and tween

136	Gisborne	Wi Pere T3 Q1	Water and tween
137	Gisborne	Wi Pere T2 Q2	Water and tween
138	Gisborne	Wi Pere T2 Q3	Water and tween
140	Wellington	Bushfield T3 Q2 S1	Water and tween
141	Wellington	Bushfield T3 Q3	Water and tween
142	Wellington	Bushfield T3 Q1	Water and tween
143	Wellington	Bushfield T2 Q2 S1	Water and tween
144	Wellington	Bushfield T1 Q3	Water and tween
145	Waikato	Cussen Rd T1 Q1	Water and tween
146	Waikato	Cussen Rd Extra 2	Water and tween
147	Waikato	Cussen Rd Extra 3	Water and tween
148	Waikato	Cussen Rd Extra 1	Water and tween
154	Waikato	Ruakura T3 Q2	Water and tween
155	Waikato	Ruakura T2 Q3	Water and tween
156	Waikato	Ruakura T3 Q1	Water and tween
157	Waikato	Ruakura T1 Q1 S1	Water and tween
158	Waikato	Ruakura T1 Q2	Water and tween
159	Waikato	Ruakura T1 Q2 S2	Water and tween
160	Waikato	Ruakura T2 Q2 S1	Spores
161	Waikato	Ruakura T2 Q1 S7	Water and tween
162	Waikato	Ruakura T2 Q1 S9	Water and tween
163	Waikato	Ruakura T2 Q1 S8	Water and tween
164	Waikato	Ruakura T2 Q1 S2	Water and tween
165	Waikato	Ruakura T2 Q1 S5	Water and tween
166	Waikato	Ruakura T2 Q1 S1	Water and tween
167	Waikato	Ruakura T2 Q1 S3	Water and tween
168	Waikato	Ruakura T2 Q1 S6	Water and tween
169	Waikato	Ruakura T2 Q1 S4	Water and tween
170	Hawkes Bay	Wai iti Rd T2 Q1	Spores
171	Hawkes Bay	Wai iti Rd T1 Q3	Water and tween
172	Hawkes Bay	Wai iti Rd T2 Q2 S1	Water and tween
173	Hawkes Bay	Wai iti Rd T3 Q2	Water and tween
174	Hawkes Bay	Wai iti Rd T3 Q3	Water and tween
175	Hawkes Bay	Wai iti Rd T1 Q2	Water and tween
176	Hawkes Bay	Wai iti Rd T1 Q1	Water and tween
177	Wellington	Bennett's Hill T3 Q3	Water and tween
178	Wellington	Bennett's Hill T3 Q2	Water and tween
179	Wellington	Bennett's Hill T2 Q2 S1	Water and tween
180	Wellington	Bennett's Hill T1 Q3	Water and tween
181	Wellington	Bennett's Hill T1 Q1	Water and tween
182	Wellington	Bennett's Hill T2 Q1	Spores
183	Wellington	Bennett's Hill T3 Q1	Water and tween

A.2 OPM primers and their sequences

OPM Primer	Sequence
OPM 1	GGTT GGT GGC T
OPM 2	ACA ACG CCT C
OPM 3	GGG GGA TGA A
OPM 4	GGC GGT TGT C
OPM 5	GGG AAC GTG T
OPM 6	CTG GGC AAC T
OPM 7	CCG TGA CTC A
OPM 8	TCT GTT CCC C
OPM 9	GTC TTG CGG A
OPM 10	TCT GGC GCA C
OPM 11	GTC CAC TGT G
OPM 12	GGG ACG TTG G
OPM 13	GGT GGT CAA G
OPM 14	AGG GTC GTT C
OPM 15	GAC CTA CCA C
OPM 16	GTA ACC AGC C
OPM 17	TCA GTC CGG G
OPM 18	CAC CAT CCG T
OPM 19	CCT TCA GGC A
OPM 20	AGG TCT TGG G

Appendix B

B.1 *Puccinia punctiformis* concentration in nine individual plants

Plant 1

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping
LM 1	4064	362	1.19×10^6	A
LT 1	2218	101	6.49×10^5	A
LM 2	1833	160	5.36×10^5	A
LT 2	1803	182	5.27×10^5	A
LM 3	1301.3	34.6	3.81×10^5	A
LT 3	992.5	35.1	2.90×10^5	A
S 1	535.5	35.9	1.57×10^5	A B
S 2	70.5	21.8	2.06×10^4	B C
S 3	52.1	14.3	1.52×10^4	B C
LB 3	31.1	30.8	9.10×10^3	C D
LB 1	3.73	2.63	1.09×10^3	D
LB 2	0.0667	0.0667	1.95×10	D

Plant 2

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping
LT 1	1758	123	5.14×10^5	A
LT 2	587.9	52.3	1.72×10^5	A B
LT 3	209.3	38	6.12×10^4	B
S 2	153.53	7.07	4.49×10^4	B C
S 3	39.3	10.2	1.15×10^4	C D
S 1	20.6	4.24	6.03×10^3	D E
LB 3	27.9	19.8	8.16×10^3	D E
LB 1	5.53	1.03	1.62×10^3	E F
LM 3	4.733	0.811	1.38×10^3	E F
LM 1	2.33	1.27	6.82×10^2	F G
LB 2	1.13	0.0667	3.31×10^2	F G
LM 2	0	0	0	G

Plant 3

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping
LM 1	7.4	5.3	2.16×10^3	A
LM 2	1.93	1.83	5.65×10^2	A B
LB 3	0.6	0.115	1.76×10^2	A B
LT 2	0.733	0.533	2.14×10^2	A B
LT 3	0.0667	0.667	1.95 x 10	B
LB 2	0	0	0	B
S 1	0	0	0	B
S 3	0	0	0	B
LB 1	0	0	0	B
S 2	0	0	0	B
LT 1	0	0	0	B
LM 3	0	0	0	B

Plant 4

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping
LT 1	3920	824	1.15×10^6	A
LT 2	1217	104	3.56×10^5	A B
LT 3	325.9	31.4	9.53×10^4	A B C
S 1	106.8	10.2	3.12×10^4	B C D
LM 3	45.33	6.01	1.33×10^4	C D E
LB 1	66.7	38.7	1.95×10^4	C D E
LM 2	35.4	3.47	1.04×10^4	C D E F
S 3	34.93	8.21	1.02×10^4	C D E F
LB 3	26.3	19.9	7.69×10^3	D E F
LM 1	10.87	4.78	3.18×10^3	D E F
LB 2	4.267	0.769	1.25×10^3	E F
S 2	5.87	5.77	1.72×10^3	F

Plant 5

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping		
LM 1	101.67	9.62	2.97×10^4	A		
LM 2	42.8	13.2	1.25×10^4	A		
LM 3	21.4	3.5	6.26×10^3	A	B	
LB 3	3.733	0.769	1.09×10^3		B	C
LB 1	7.8	7.5	2.28×10^3			C
LB 2	2	0.462	5.85×10^2			C
S 1	0.867	0.667	2.54×10^2			C
S 3	0	0	0			C
LT 2	0.667	0.667	1.95×10^2			C
LT 1	0.667	0.667	1.95×10^2			C
S 2	0.0667	0.0667	1.95×10			C
LT 3	0	0	0			C

Plant 6

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping		
LB 3	44.0	13.8	1.29×10^4	A		
LB 2	21.33	6.52	6.24×10^3	A		
LT 1	16.53	4.67	4.84×10^3	A		
LM 1	10.47	2.45	3.06×10^3	A	B	
LM 2	10.4	3.36	3.04×10^3	A	B	
LT 2	9.27	3.38	2.71×10^3	A	B	
LM 3	9.00	3.61	2.63×10^3	A	B	
LT 3	2.8	1.63	8.19×10^2		B	C
S 1	0.667	0.481	1.95×10^2			C
LB 1	0.400	0.200	1.17×10^2			C
S 2	0	0	0			C
S 3	0	0	0			C

Plant 7

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping
LM 2	1449	30.3	4.24×10^5	A
LM 3	1239	252	3.62×10^5	A B
LT 1	715.3	43.6	2.09×10^5	A B
LT 2	140.9	30.3	4.12×10^4	A B C
LT 3	103.9	3.51	3.04×10^4	A B C
S 1	64.4	302	1.88×10^4	A B C D
LM 1	407	400	1.19×10^5	B C D E
LB 3	18.13	9.36	5.30×10^3	C D E
S 2	13.33	3.7	3.90×10^3	C D E
S 3	20.5	10.2	6.00×10^3	C D E
LB 2	2.27	1.47	6.64×10^2	D E
LB 1	0.400	0.400	1.17×10^2	E

Plant 8

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping
LM 3	3170	163	9.27×10^5	A
LT 2	2916	111	8.53×10^5	A
LT 1	2854	187	8.35×10^5	A
LM 2	1461	703	4.27×10^5	A
LT 3	1795	867	5.25×10^5	A B
S 3	429	322	1.25×10^5	A B C
S 2	152.9	21.4	4.47×10^4	A B C D
S 1	145.7	33.4	4.26×10^4	A B C D
LM 1	383	382	1.12×10^5	B C D E
LB 3	2.6	1.91	7.61×10^2	C D E
LB 2	2.6	1.91	7.61×10^2	D E
LB 1	0.2667	0.0667	7.80×10	E

Plant 9

Location	Original data (ng/µl)	SE Mean	Spores/µl	Grouping			
LB 2	2627	216	7.68×10^5	A			
LB 1	2915	1548	8.53×10^5	A			
LM 3	2215	428	6.48×10^5	A			
LT 1	1929	634	5.64×10^5	A	B		
LT 3	1686	652	4.93×10^5	A	B		
LM 2	1310	334	3.83×10^5	A	B		
LT 2	1088	569	3.19×10^5	A	B		
LM 1	546.3	57.4	1.60×10^5	A	B	C	
LB 3	619	374	1.81×10^5	A	B	C	D
S 1	214.2	57.2	6.27×10^4		B	C	D
S 2	62.5	11.6	1.83×10^4			C	D
S 3	45.93	1.87	1.34×10^4				D

Appendix C

C.1 Potting mix recipe

500 L of Potting mix contained 400L composted bark, 100L pumice, 1500g Osmocote extract (16-3.9-10 NPK), 500g horticultural lime and 500g hydraflo (wetting agent).

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